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INTERACTION OF SMOOTH MUSCLE CALPONIN WITH CALCIUM BINDING PROTEINS

BY

FIONA L. WILLS ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL 1994

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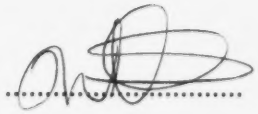

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Nurture your mind with great thoughts;
to believe in the heroic makes heroes.

Benjamin Disraeli

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***Interaction of Smooth Muscle Calponin with Calcium Binding Proteins*** *submitted by Fiona L. Wills in partial fulfillment of the requirements for the degree of Doctor of Philosophy.*

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ABSTRACT

Calponin interacts with several Ca^{2+} binding proteins in a Ca^{2+} dependent manner. In order to determine the possible biological relevance of these interactions in smooth muscle function it is necessary to characterize the strength and stoichiometry of the complexes formed. The interaction between calponin and calcium binding proteins can be monitored through an acrylodan label on cysteine 273 of calponin. This probe is very environment sensitive and responds to the interaction by the emission peak blue shifting and by the fluorescence intensity increasing at 460 nm. The stoichiometric nature of the complexes formed has been determined using analytical ultracentrifugation and is two calcium binding proteins to one calponin and the interaction is Ca^{2+} dependent. The interaction of caltropin with calponin may be biologically relevant since caltropin is isolated from smooth muscle, and shows strong affinity for calponin. Caltropin is capable of regulating calponin's inhibition of the actomyosin ATPase, and it does this more efficiently than calmodulin. Thus calponin and caltropin have the necessary properties to function as a regulatory complex in smooth muscle contraction.

In order to delineate the two sites of interaction with calcium binding proteins, fragments of calponin have been studied. One fragment comprising residues 2-51 was isolated from a CNBr cleavage of calponin. This amino terminal fragment possesses one tryptophan which can be used to monitor interactions with calcium binding proteins. A carboxyl terminal truncated mutant of calponin

comprising residues 1-228 has been produced by recombinant techniques. This 1-228 fragment has two tryptophans which can be used to monitor interactions with CaBP's. Analytical ultracentrifugation has shown that CP 1-228 is able to bind two mols of CaBP per mol of 1-228 in a Ca^{2+} dependent fashion, indicating there is a second site of interaction between residues 52-228. A second mutant produced through recombinant techniques comprises residues 45-228, and is also able to bind CaBP's. The two identified sites of interaction correspond to two exposed segments of calponin as predicted by surface plot analysis. This study represents the first attempt to characterize the interaction between calponin and the calcium binding proteins, and the findings suggest this may be an important method to regulate calponin's function in smooth muscle contraction.

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ABBREVIATIONS

acrylodan	6-acryloyl-2-(dimethylamino)napthalene
AEANA	N-(2-aminoethyl)-4-azido-2-nitroaniline
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase;
β-ME	β-mercaptoethanol
cAMP	adenosine 3', 5'-cyclic monophosphate
cGMP	guanosine 3', 5'-cyclic monophosphate
CaBP	calcium binding protein
CD	circular dichroism
CP	calponin
CT	caltropin
CNBr	cyanogen bromide
DMSO	dimethylsulfoxide
DTNB	5-5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
EDC	1-ethyl-3-[3(dimethylamino)propyl] carbodiimide
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethylether)N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
FITC	fluorescein-5-isothiocyanate
FPLC	fast protein liquid chromatography
ΔG_D	observed free energy of unfolding
$\Delta G_D^{H_2O}$	free energy of unfolding in H_20
Gdn·HCl	guanidine hydrochloride
ΔH	enthalpy difference
HMM	heavy meromyosin
HPLC	high performance liquid chromatography

K_d	dissociation constant
kDa	kilodalton(s)
MES	2-(N-morpholino)ethanesulfonic acid;
MOPS	3-(N-morpholino) propane-sulfonic acid
MLC	myosin light chain
MLCK	myosin light chain kinase
$NHS(SO_3)$	N-hydroxysulfosuccinimide
NTCB	2-nitro-5-thiocyanobenzoic acid;
pCa^{2+}	$-\log[Ca^{2+}]_{free}$;
PKC	protein kinase C
RP	reversed phase
rpm	revolutions per minute
S1	subfragment 1 of myosin
SDS-PAGE	sodium dodecyl sulfate polyacrlyamide gel electrophoresis
SMCaBP-11	smooth muscle calcium binding protein-M_r 11000 Da (caltropin)
SP	sulphopropyl
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol;
T_m	midpoint of temperature melting
TNC	Troponin C, Ca^{2+} binding subunit of troponin
TNI	Troponin I, inhibitory subunit of troponin
TNT	Troponin T, tropomyosin binding subunit of troponin
TNS	2-toluidinylnaphthalene-6-sulfonate
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
Glu	glutamic acid
Gln	glutamine
Gly	glycine
His	histidine
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Tyr	tyrosine
Trp	tryptophan
Val	valine

Chapter 1

INTRODUCTION

The study of the molecular mechanism underlying muscle contraction has led to the sliding filament model in which the formation of cycling crossbridges between actin and myosin controls force development [for review see Hartshorne (1987), Murphy (1989), Hai & Murphy (1989), Rasmussen *et al.* (1987), Marston & Smith (1985), Chacko *et al.* (1986)]. Muscle contraction is produced by the cyclic attachment and detachment of the globular heads of the myosin molecule to actin in the thin filament, which results in the sliding of the thick myosin filament past the thin actin filaments (Figure 1.1). The energy for this actin-myosin interaction is supplied by ATP which is hydrolyzed at a catalytic site on the myosin head, a process activated by actin. In the rigor state, myosin heads are bound tightly to the actin filament in the absence of nucleotide. Binding of ATP to myosin releases it from the actin filament. The myosin is able to hydrolyze the ATP to ADP and P_i which remain bound to myosin, after which the myosin binds to actin. Release of the ADP and P_i from myosin is accompanied by the power stroke in which the complex changes conformation and the thick and thin filament slide past one another. This complex is once again without nucleotide and is in the rigor state until a fresh cycle begins again.

CALCIUM REGULATION

Calcium levels in the cytoplasm of the muscle cell determine whether the

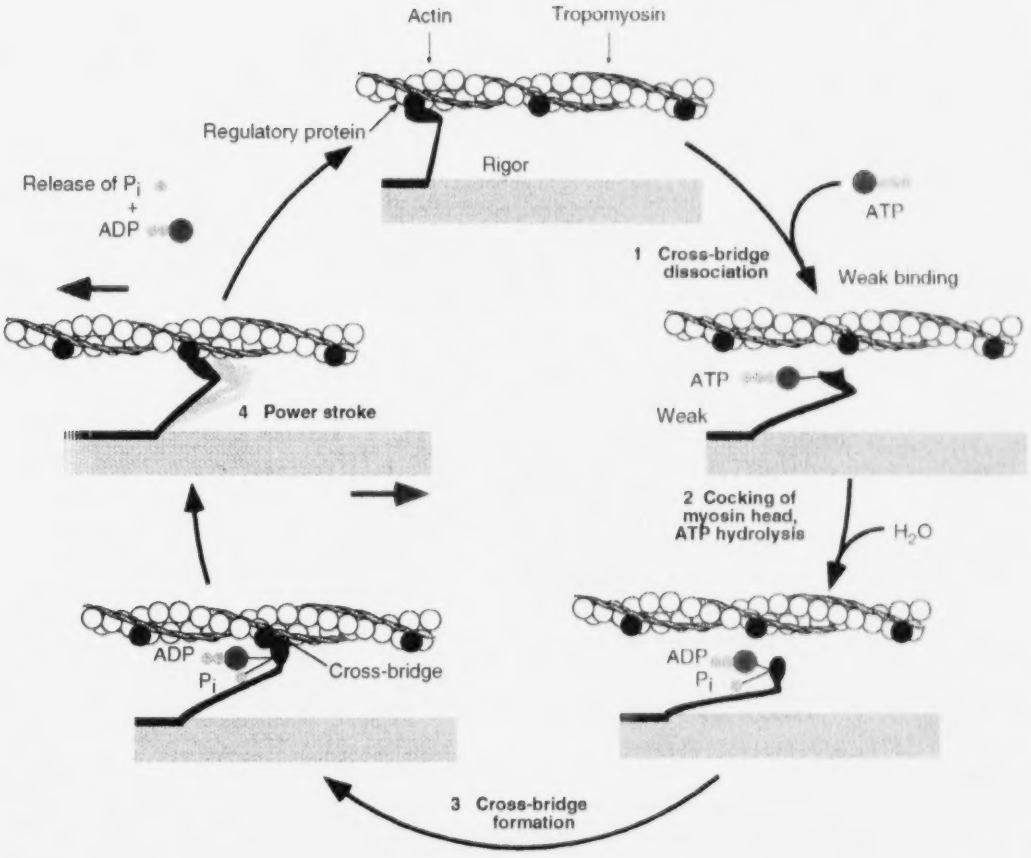

Figure 1.1: The cycle of muscle contraction showing the stages of interaction of the myosin head with the thin filament. [Adapted from Voet & Voet (1990)]

cell will contract or not. These calcium levels are in turn controlled by neural stimulation which causes calcium release from the sarcoplasmic reticulum. In skeletal and cardiac muscle the calcium interacts with troponin C and alters the conformation of the troponin complex which in turn alters its interaction with tropomyosin (Rasmussen *et al.*, 1987). In resting muscle the tropomyosin-actin interaction is such that interaction between actin and myosin is inhibited. Upon binding of calcium to troponin C the change in conformation of troponin C causes tropomyosin to change its position, allowing the release of inhibition such that actin forms crossbridges with myosin causing the activation of MgATPase and the muscle contracts. Thus in skeletal and cardiac muscle muscle contraction is a thin filament regulated process.

Calcium also controls smooth muscle contraction; however the most accepted mode of regulation elucidated to date does not involve a thin filament on/off switch for the actin-myosin crossbridge as in skeletal muscle. Smooth muscle does not contain the troponin complex, and regulation of the contraction cycle in smooth muscle is known to occur at the level of the thick filament by phosphorylation of the myosin light chain which promotes the activity of the actomyosin ATPase (Adelstein & Eisenberg, 1980).

In addition to the mechanistic control of smooth muscle contraction, it also does not appear that stimulation occurs in the same manner as skeletal muscle. As well as membrane depolarization through autonomic neural control, hormones such as norepinephrine, vasopressin, and angiotensin, may also be able to bring

about contraction either simultaneously, or without concomitant membrane depolarization [Rasmussen *et al.* (1987), Van Breeman & Saida (1989)]. In addition to the release of calcium from intracellular stores, upon stimulation of a smooth muscle cell, an influx of calcium across the plasma membrane may be an important regulatory event, and may occur via potential dependent and/or receptor dependent calcium channels (figure 1.2). In support of this idea, while removal of extracellular calcium causes little change in the ability of skeletal muscle to contract, in smooth muscle it does lead to a decrease in the ability to function.

There are a range of extracellular signals (peptide hormones, neurotransmitters, growth factors, etc.) which are able to increase intracellular calcium levels by acting via stimulation of membrane phosphoinositide metabolism. Phospholipase C catalyzes hydrolysis of phosphotidylinositol 4,5-bisphosphate, to generate two second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Berridge, 1984, 1987). IP3 induces calcium release from the sarcoplasmic reticulum (Somlyo & Somlyo, 1990) while DAG remains membrane bound due to its lipophilic character and serves to activate the calcium and phospholipid dependent protein kinase C. The effects of PKC on contraction are still being investigated - phorbol esters, substitutes for DAG in PKC activation may alternatively stimulate or inhibit contraction and may increase calcium sensitivity. PKC may indirectly affect intracellular calcium concentrations in addition to the direct phosphorylation of proteins involved in contractile regulation, and thus the precise mechanism of action is not easily determined (Kamm & Stull, 1989).

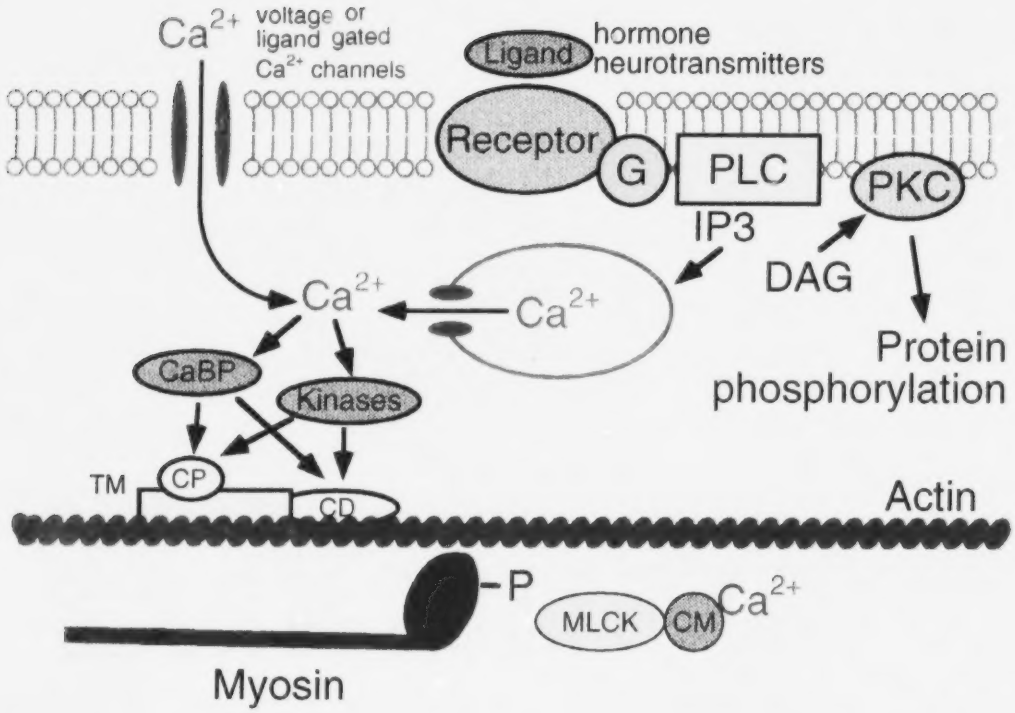

Figure 1.2: Model of the signalling pathways through which the contraction cycle of smooth muscle may be regulated. CP, calponin; CD, caldesmon; PLC, phospholipase C; IP3, inositol triphosphate; TM, tropomyosin; PKC, protein kinase C; CM, calmodulin; MLCK, myosin light chain kinase; DAG, diacylglycerol; CaBP, calcium binding protein. [Adapted from Gerthoffer (1991)].

CONTRACTILE PROTEINS

The fundamental proteins involved in the contractile apparatus, are the same in both smooth and skeletal muscle, although there are some subtle differences (Marston & Smith, 1985). Smooth muscle actin has the same molecular mass of 42 kDa as skeletal muscle actin, but differs slightly in amino acid sequence in the amino terminal of the molecule, but to date these changes have no known functional significance. Tropomyosin also has the same molecular mass of 33 kDa / chain with some amino acid differences from that found in skeletal muscle. These differences are mostly located in the carboxyl terminal portion and in the region of the molecule which in skeletal muscle interacts with troponin T. Smooth muscle tropomyosin will still bind troponin and function in a skeletal muscle ATPase system, however the interaction with troponin is modified (Hartshorne, 1987). The role of tropomyosin in smooth muscle is poorly understood. While it is well documented that tropomyosin plays a fundamental role in skeletal muscle, in smooth muscle, tropomyosin has not been assigned a role as a necessary component of thin filament linked regulation, although it is required for full activation of smooth muscle actomyosin (Chacko, 1981). Tropomyosin will not enhance the ATPase of unphosphorylated smooth muscle myosin, however it will enhance sensitivity of the ATPase to the level of phosphorylation. In the absence of tropomyosin, a threshold of 60% phosphorylation of MLC 20 exists before

ATPase occurs, however, in the presence of tropomyosin that threshold drops to only 10% which is closer to the situation observed in native muscle. Thus tropomyosin seems to play a role in modulating the phosphorylation-ATPase relationship (Merkel *et al.*, 1984). The role tropomyosin may play in regulation along with other thin filament proteins is yet to be established.

Although smooth muscle myosin closely resembles that of skeletal muscle, it differs functionally. The heavy chain has a mass of 200 kDa, as in skeletal muscle, however the light chain composition is different. There are two sets of light chains, the regulatory light chains of molecular mass 20 kDa, and the essential light chains of 17 kDa. The level of ATPase activity even under optimal conditions is ten fold lower than in skeletal muscle, and smooth muscle myosin is subject to more direct regulation by calcium in that the regulatory light chains require calcium levels in the cell to be elevated in order to be phosphorylated. This dependence upon phosphorylation is similar to myosins of non-muscle cells, where interaction with actin, and contraction, occur only when the light chains are phosphorylated. These differences in myosin function are not haphazard but instead reflect the different role that smooth muscle plays. There are differences between the responses to stimulation by smooth and skeletal muscle. Skeletal muscle normally responds with repetitive contractile activity to repetitive neural stimulation. Many smooth muscles display either a biphasic response involving an initial transient increase in force followed by a more slowly developing sustained contraction, or alternatively a monotonic increase in contraction to a sustained

plateau. In skeletal muscle the relationship between strength and duration of contraction with the change in the intracellular free calcium is direct. This is not the case in smooth muscle where a current area of research is how tension is maintained even when cytosolic calcium levels have dropped (reviewed in Hai & Murphy, 1989). Rather than being required to quickly contract repeatedly, smooth muscle surrounds internal organs, and is required to maintain tension for long periods of time. The mechanism of contraction and force maintenance in smooth muscle is therefore specifically adapted to provide tension for long periods while hydrolyzing 5-10 times less ATP than skeletal muscle would performing the same task.

ULTRASTRUCTURE

Smooth muscle contains relatively small mononucleated cells that are coupled to one another both electrically and mechanically [Hartshorne (1987), Rasmussen *et al.* (1987), Marston & Smith (1985)]. The thick and thin filaments are not found in the strictly ordered sarcomeres that result in the striations so characteristic of skeletal muscle but absent in smooth muscle. One end of the thin filament is attached to a dense body. The dense bodies may be found in the cytoplasm (cytoplasmic dense bodies CDB) or attached to the membrane (membrane bound dense bodies MBDB). These dense bodies are believed to be analogous to the Z lines of striated muscle serving to anchor the thin filaments. This comparison

has been made because both Z lines and dense bodies contain α-actinin, a 100 kDa protein functional as a homodimer, which binds actin and can cause gelation of F-actin by crosslinking it. Its interaction with actin can be inhibited by tropomyosin and its crosslinking activity can be inhibited by vinculin. (reviewed by Marston & Smith, 1985). It has also been shown that thin filaments on either side of the CDB possess different polarity just as the filaments have opposite polarity on either side of the Z line in skeletal muscle. While α-actinin is present in both CDB and MBDB, another protein, vinculin, a 130 kDa protein, is present only in MBDB and can cause a small increase in F-actin viscosity. Vinculin is believed to function in anchoring the α-actinin to the membrane, along with other candidate proteins, meta-vinculin and talin. Together it is believed they may function like the spectrin - ankyrin - band 3.1 - actin membrane attachment system (reviewed in Marston & Smith, 1985).

Small *et al.* (1986) proposed from immunocytochemistry of ultrathin sections that two distinct actin-containing domains exist in smooth muscle: an actomyosin domain composed of continuous longitudinal arrays of actin and myosin filaments, and an actin-intermediate filament domain that forms longitudinal fibrils containing actin, filamin, desmin, and α-actinin rich dense bodies, but which are free of myosin [Hartshorne (1987), Rasmussen *et al.* (1987), Marston & Smith (1985)]. While these intermediate filaments are found in many eukaryotic cells, they are particularily numerous in smooth muscle cells. The function of these intermediate filaments has not been established; however, it is not thought that they play a

direct role in muscle contraction in modifying ATP hydrolysis or myosin function. It currently is believed they may contribute in an indirect fashion by joining the contractile units of the cell through the dense bodies and forming a cytoskeletal network that would distribute tension evenly throughout the cell (Hartshorne, 1987). If these filaments were to play a role in tension maintenance then any of a number of proteins involved in this cytoskeletal network could be controlled, for example, by phosphorylation and this could be an alternate form of regulation (Kamm & Stull, 1989). The hypothesis of a relationship between intermediate filament phosphorylation and sustained contractions still needs to be tested.

In the contractile domain the thick and thin filaments of the smooth muscle cell interdigitate just as in skeletal muscle. There is a far greater ratio of actin to myosin in smooth muscle compared to skeletal which can be accounted for in part by the presence of actin in the intermediate filaments, but also in a lower stoichiometry of thick to thin filaments [Hartshorne (1987), Rasmussen *et al.* (1987), Marston & Smith (1985)]. There tend to be up to 15 thin filaments associated with one thick filament either in a rosette formation or in random arrangement. The thin filaments are arranged in parallel tracts along the longitudinal axis of the cell with regular 11 μM spacing between filaments. Just as in skeletal muscle, contraction does not involve a change in length of the filament, but rather a change in the degree of overlap between thick and thin filament.

The regulation of interaction between the thick and thin filaments of smooth muscle has many questions still to be resolved. The role of phosphorylation of

myosin light chain in contraction has been established, however, its precise role and the contribution of other regulatory mechanisms has still to be established.

REGULATION BY MYOSIN LIGHT CHAIN PHOSPHORYLATION

Phosphorylation of the light chains of myosin is believed to be the primary form of regulation of contraction in smooth muscle cells [Hartshorne (1987), Hai & Murphy (1989), Chacko *et al.* (1986)]. Upon stimulation, intracellular calcium concentrations rise from 10^{-7}M to 10^{-6}M resulting from the release of calcium from the sarcoplasmic reticulum and an influx of extracellular calcium. This calcium binds to calmodulin inducing a conformational change promoting the interaction of calmodulin with myosin light chain kinase (MLCK) (figure 1.3). This interaction activates MLCK so that it phosphorylates the 20 kDa regulatory light chain of myosin at serine 19, which in turn causes a conformational change of myosin at the S1/S2 junction and allows for interaction with actin, activation of the myosin MgATPase, and crossbridge cycling. Crossbridge cycling continues for as long as calcium levels remain elevated, however, when the intracellular concentration of calcium returns to basal levels calcium dissociates from calmodulin. Apo calmodulin no longer binds to and activates MLCK so light chain phosphorylation ends. Phosphatases, which are always present in the cell, begin to dominate as the kinase no longer functions, and the light chains are dephosphorylated eliminating interaction with actin and ATPase activity. An additional level of

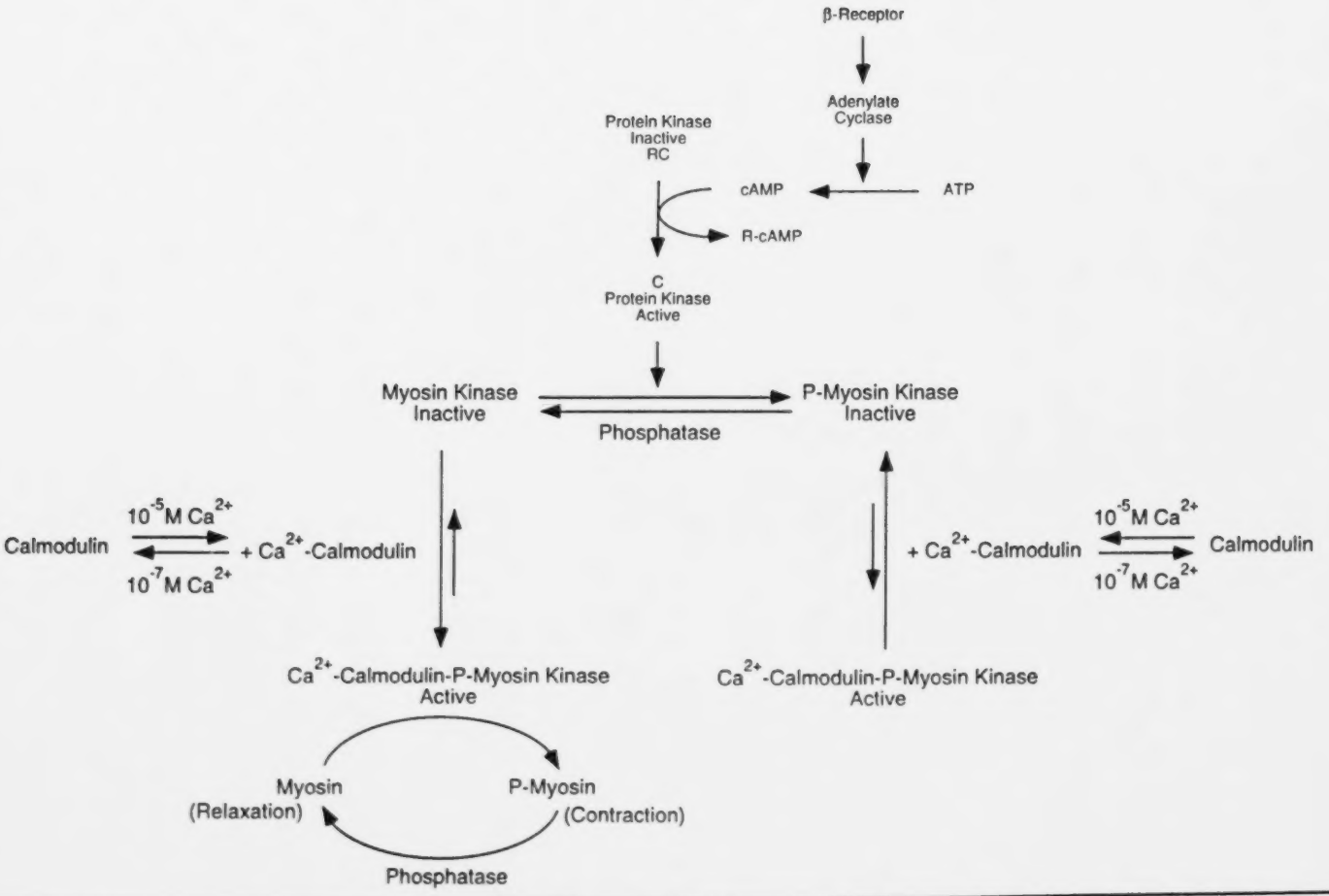

Figure 1.3: Regulation of smooth muscle contraction through myosin light chain phosphorylation, and activation of myosin light chain kinase activity.

regulation is that MLCK can be converted to an inactive form with weak affinity for Ca2+/calmodulin by phosphorylation with cyclic AMP dependent protein kinase (figure 1.3). This kinase activity is stimulated by the β-adrenergic receptor and explains how a substance such as epinephrine which binds to this receptor is able to cause muscle relaxation. When MLCK is phosphorylated by cAMP dependent protein kinase, it no longer binds to calmodulin and no longer phosphorylates myosin light chain leading to inhibition of the ATPase activity and loss of crossbridge cycling (Hartshorne, 1987).

Force was typically thought to be proportional to myosin phosphorylation in smooth muscle. For the MLCK model to account for sustained contraction two assumptions must be made. First, as long as the muscle remains contracted the intracellular free calcium levels must remain elevated, and second, as long as the muscle remains contracted the content of phosphorylated myosin light chain must remain high. Neither of these assumptions has proven to be correct however (Rasmussen *et al.*, 1987).

Upon stimulation there is an immediate and rapid rise in calcium concentration in the cell which is a transient rise lasting only a few minutes (figure 1.4). The calcium level then returns to near basal levels even though there is still sustained contraction. The phosphorylated myosin light chain content also rises initially with calcium concentration, but it then falls off slowly over a period of 15-30 minutes to values that are not distinguishable from basal levels, once again while there is sustained contraction of the muscle cell. It has been shown that concomitant with

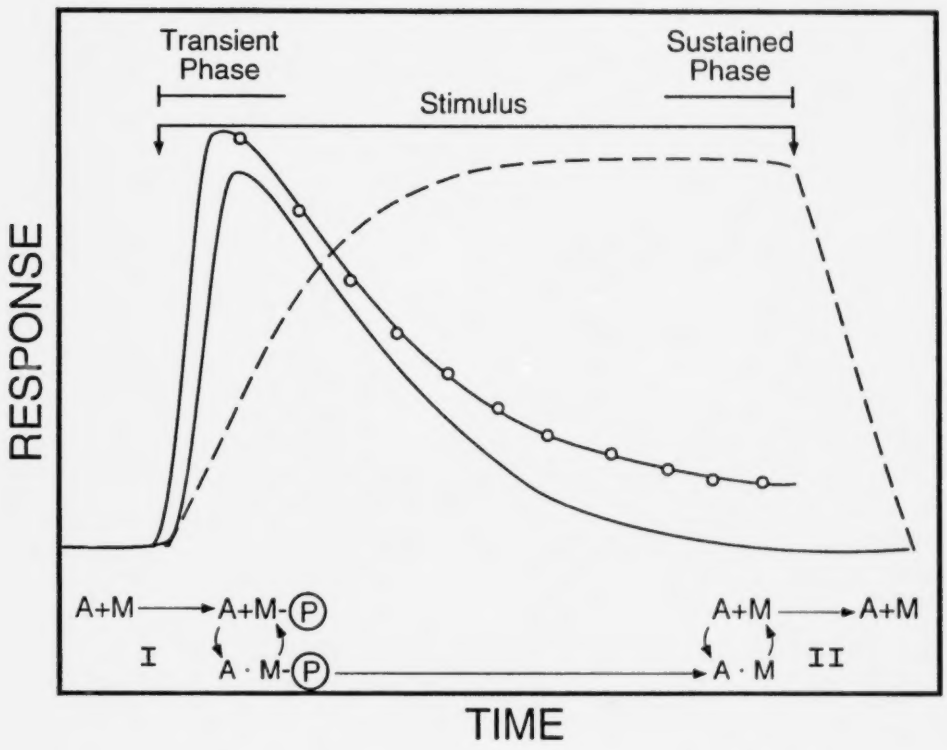

Figure 1.4: Model of the calcium concentration and shortening velocity (open circles), myosin light chain phosphorylation (solid line), and force (dashed line) relationships during the course of smooth muscle contraction. Stage I involves rapid cycling of phosphorylated crossbridges which is controlled by phosphorylation of myosin light chains. Stage II is proposed to involve slowly or non-cycling crossbridges, the control mechanism of which is yet to be resolved. A, actin; M, myosin. [Adapted from Kamm & Stull (1985)].

the calcium level decrease in the cell there is a drop in phosphorylation of myosin light chains, which does not necessarily result in a decrease in tension of the muscle (reviewed in Hai & Murphy, 1989). In addition, smooth muscle thin filaments, used in conjunction with skeletal myosin (Marston & Smith, 1984), or thiophosphorylated smooth muscle myosin (Marston & Lehman, 1985) are still calcium sensitive, which indicates the thin filaments of smooth muscle must possess a calcium sensitive component, the identity of which is yet to be established. The model for smooth muscle regulation had to be expanded, therefore, to include other modulatory pathways in order to explain the observed phenomena. A number of other possibilities have been explored. It is apparent, however, that additional control of muscle contraction occurs at the level of the thin filament (Marston & Smith, 1985).

ALTERNATIVE REGULATORY PATHWAYS

One possibility for the regulation of myosin is direct binding of calcium to myosin [Hartshorne (1987), Kamm & Stull (1989)]. This would not be unprecedented since molluscan muscle is regulated in this manner. There is evidence that calcium may increase the actin activated MgATPase of phosphorylated smooth muscle myosin; however, the calcium concentrations required for this activation are much greater than those required to saturate calmodulin and to activate MLCK. Furthermore, these levels are higher than those

observed in the smooth muscle cytoplasm. It seems unlikely therefore that calcium binding to myosin has physiological relevance.

Phosphorylation of the myosin heavy chain is another possibility for regulation of contraction (Kamm & Stull, 1989). In non-muscle cells the myosin heavy chain can be phosphorylated and this phosphorylation diminishes actin-myosin interaction and decreases assembly of myosin filaments. Myosin from molluscan smooth muscle is phosphorylated in the rod portion of the heavy chain by an endogenous unidentified kinase and correlates with the transition from the catch state to relaxation in this muscle. Kawamoto and Adelstein (1988) reported myosin heavy chain (MHC) phosphorylation in aortic smooth muscle tissue or cells in culture, and it will be of interest in the future to learn if this has a role in contraction regulation.

THE LATCH BRIDGE HYPOTHESIS

The original tenets of the latch bridge theory are that initiation of contraction results from formation of cycling actin-myosin crossbridges which requires phosphorylation of the myosin light chain. However, when a phosphorylated myosin light chain involved in a crossbridge was dephosphorylated a new type of long lasting actin-myosin bridge formed - the latch bridge [Hartshorne (1987), Murphy (1989), Hai & Murphy (1989), Rasmussen *et al.* (1987)]. In experiments correlating phosphorylation of the myosin light chain with contraction, Dillon &

Murphy (1982) found a decrease in phosphorylation prior to achievement of full force, but observed a strong correlation between light chain phosphorylation and shortening velocity. They made the assumption that load bearing capacity was proportional to the number of attached crossbridges, and that velocity provides an estimate of crossbridge cycling rate. The initial response to stimulation is consistent with the hypothesis that activation of the contractile apparatus involves an obligatory calcium stimulated phosphorylation of the LC 20; however shortening velocity decreases with time although the numbers of crossbridges generating force remains constant. This indicated that crossbridge cycling rate depended upon the phosphorylation of MLC. In the model LC 20 phosphorylation would correlate closely with ATPase activity and shortening velocity. The second part of the hypothesis is that crossbridges can remain attached and generating force even after the phosphorylated fraction of myosin capable of cycling decreases to about 25%. Assuming that the MLCK - myosin light chain phosphatase system can act on attached as well as on free crossbridges, it is possible that dephosphorylation of an attached crossbridge could occur resulting in the arrest of crossbridge cycling. Such an attached, slowly cycling, crossbridge was termed a latch bridge, which is capable of maintaining force while acting as a load on the remaining crossbridges producing a decrease in velocity with increase in force (Dillon *et al.*, 1981). The cellular conditions which would cause these bridges to form is not known; however in order to explain the data the latch bridges would be sensitive to calcium and would only form after the concentration of calcium fell back close

to basal levels.

Implicit in this latch bridge theory is that both unloaded shortening velocity and ATPase activity are directly correlated with the MLC 20 phosphorylation level, and several reports have been published where shortening velocity and ATPase activity change independently of LC 20 phosphorylation [Haeberle *et al.* (1985), Siegman *et al.* (1984), Moreland *et al.* (1986), Kenney *et al.*(1990)]. Thus the latch bridge theory in its original form is also unable to explain the observed behavior of smooth muscle regulation. Finding an explanation for these phenomena is a major thrust of current research. In view of the myosin light chain phosphorylation theory being unable to explain all aspects of smooth muscle contraction, alternative regulatory possibilities must be explored.

THIN FILAMENT LINKED REGULATION

Much work has been done trying to find a thin filament linked regulatory system in smooth muscle as in skeletal muscle and there is evidence to suggest one exists. Several authors have demonstrated the decoupling of muscle tensions from the thick filament linked regulation by myosin phosphorylation [Rasmussen *et al.* (1987), Kamm & Stull (1989), Gerthoffer (1987), Tansey *et al.* (1990)], and it has also been found that thin filaments isolated from smooth muscle are able to activate both skeletal myosin and thiophosphorylated smooth muscle myosin in a calcium dependent manner [Marston & Smith (1984), (1985), Marston & Lehman

(1985)]. When skeletal myosin is used with purified actin and tropomyosin no such activation occurs showing there must be an additional element in the smooth muscle thin filament conferring this sensitivity. Several proteins have been isolated from smooth muscle thin filaments in order to identify the additional regulatory component.

LEIOTONIN

Leiotonin is a thin filament regulatory system originally proposed by Ebashi and colleagues (1980). The theory suggests that actin and tropomyosin are normally not able to activate smooth muscle myosin and that activation of leiotonin by calcium leads to ATPase activity in the absence of myosin light chain phosphorylation [Hartshorne (1987), Marston & Smith (1985)]. Leiotonin was proposed to contain two subunits, one of 80 kDa termed leiotonin A, and a calcium binding subunit of 18 kDa termed leiotonin C. Leiotonin was proposed to be present in a 1:100 ratio to actin suggesting it could not have a structural role similar to that of troponin while present in such low concentrations. Its mechanism of action remains unknown and more information is required to confirm the activity of leiotonin. Other labs were unable to reproduce the results of Ebashi, and it has been proposed that leiotonin preps were actually contaminated by MLCK since all of the results could be explained on this basis, and leiotonin had no effect on thiophosphorylated myosin.

CALDESMON

A component of the smooth muscle thin filament, of molecular mass 120-150kDa, was first described by Sobue *et al.* (1981) which was capable of inhibiting the ATPase activity of actomyosin in a Ca2+ dependent fashion [Hartshorne (1987), Marston & Smith (1985), Kamm & Stull (1989), Sobue *et al.* (1982)]. The molecular weight from the amino acid sequence has since been found to be 87kDa; the anomolous behavior on SDS gels has been attributed to poor SDS binding due to its acidic nature (Bryan, 1990). Two forms of caldesmon have been identified by immunological methods, a high molecular weight and a low molecular weight isoform which retains the actin, calmodulin and myosin binding properties of the high molecular weight form, but is missing a central repeating region known to be highly helical (Matsumura & Yamashiro, 1993). The low molecular weight variant is particularly abundant in cell cultures (Bretscher & Lynch, 1985), while the predominant form expressed in smooth muscle cells is the high molecular weight form. Isolated caldesmon is highly asymmetric with a length of 74 nm and width of 1.9 nm (Graceffa *et al.*, 1988). Caldesmon binds to calmodulin in a calcium dependent manner, while it binds to actin, tropomyosin and myosin in a calcium independent manner. Caldesmon binds to actin-tropomyosin with higher affinity than to actin alone (Velaz *et al.*, 1989) presumably due to additional interactions with tropomyosin as well as actin. Tropomyosin is not required for the inhibitory

activity, although tropomyosin does enhance the activity of caldesmon. The requirement for tropomyosin is dependent on the conditions used, being determined by ionic strength, concentration of actin, concentration of caldesmon, and whether myosin or soluble myosin subfragments are employed (Chalovich *et al.*, 1990). Caldesmon binds to calmodulin, and not actin, in the presence of calcium, and binds to F-actin, not calmodulin, in the absence of calcium. This led to the proposal of a flip-flop mechanism for calcium regulation of caldesmon binding to thin filaments, however this mechanism has since been disputed because the concentration of calmodulin required to reverse the ATPase activity of actomyosin is lower than that required to reverse the binding of caldesmon to actin. This was found in both gizzard and aorta tissue and suggested that reversal of inhibition of actomyosin ATPase activity does not occur strictly by a reversal of the binding of caldesmon to actin, but rather an inactive complex of calcium-calmodulin-caldesmon-actin may form [(Horiuchi *et al.* (1986), Smith *et al.* (1987), Pritchard & Marston (1989)]. Ca2+/calmodulin ratios in excess of those found physiologically are required to reverse the inhibition by caldesmon. This has led to the suggestion that the *in vivo* calcium binding factor which controls caldesmon function has yet to be identified. One alternative calcium binding protein which has been proposed to regulate caldesmon at physiologically reasonable concentrations is caltropin originally isolated by Mani & Kay (1990). Research is currently ongoing to determine the role the caldesmon-caltropin interaction may play in smooth muscle regulation.

Ikebe & Reardon (1988) observed that caldesmon can crosslink actin to myosin. The caldesmon binding sites were localized to the S2 region of myosin (Bartegi *et al.*, 1990). Since the actin, calmodulin, and tropomyosin binding sites are all located within the carboxyl terminal region of caldesmon [Szpacenko & Dabrowska (1986), Fujii *et al.* (1987), Makuch *et al.* (1989), Riseman *et al.* (1989)], whereas the myosin binding site is near the amino terminus [Sutherland & Walsh (1989), Velaz *et al.* (1990)] it has been suggested that caldesmon can tether myosin to actin and work is ongoing to determine if these interactions could contribute to the latch state of smooth muscle.

Caldesmon phosphorylation may also play a regulatory role [Hartshorne (1987), Kamm & Stull (1989)]. Caldesmon is phosphorylated by a co-purifying Ca2+/calmodulin dependent kinase and only the unphosphorylated form is inhibitory (Ngai & Walsh, 1985). It has also been shown that caldesmon is phosphorylated *in vitro* by PKC at the carboxyl terminal domain [Vorotnikov *et al.* (1988), Tanaka *et al.* (1990)] and that ATPase inhibition was proportional to the phosphorylation level (Tanaka *et al.*, 1990). Incorporation of phosphate into the amino terminal domain can also inhibit interaction with myosin (Sutherland & Walsh, 1989). Since caldesmon can be phosphorylated *in vitro* by a number of kinases, and the *in vivo* phosphorylation sites have yet to be confirmed, there are still many questions to be addressed before assigning a functional role to phosphorylation of caldesmon in regulating ATPase (Marston & Redwood, 1991). It has also been suggested that phosphorylation may play a role in cell cycling

regulation. This is suggested by the observation that cdc2 kinase phosphorylates the caldesmon low molecular weight variant *in vivo* during mitosis, and phosphorylation is accompanied by dissociation of caldesmon from microfilaments (Yamashiro *et al*., 1990) The high molecular weight isoform has also been shown to be a substrate for cdc2 kinase *in vitro* [Mak *et al*. (1991), Yamashiro *et al*. (1991)].

Chalovich *et al*. (1990) and Horiuchi and Chacko (1989) found the carboxyl terminal fragment of caldesmon reduced $K_{binding}$ with no concomitant effect upon V_{max} for the actin activated ATPase activity of HMM. *In vitro* motility studies showed that the carboxyl terminal region of caldesmon did not affect velocity of actin filaments over immobilized myosin (Haeberle *et al*. 1992), a result which was independent of the amount of bound myosin. Whole caldesmon was also unable to affect filament velocity, except under conditions where it could oxidize and crosslink filaments. In a parallel study, Shirinsky *et al*. (1992) showed caldesmon inhibited movement in a graded fashion, however they were using 1mM DTT at which concentration Haeberle *et al*. (1992) also saw inhibition of movement which they attribute to actin crosslinking. The conclusion drawn was that as long as the actin filament can contact myosin, the actual number of cycling crossbridges does not affect velocity. Thus the binding of caldesmon to actin does not alter the rate of crossbridge cycling indicating that the actin caldesmon, myosin complex is not a load bearing structure. Haeberle *et al*. (1992) did find that whole caldesmon increased the binding of actin filaments to myosin, whereas the carboxyl terminal

fragment was unable to do this, supporting the idea that caldesmon can act as a tether between actin and myosin.

Preparations of chicken gizzard thin filaments have been shown to contain calponin in addition to actin, tropomyosin, and caldesmon (Nishida *et al.*, 1990). Calponin and caldesmon demonstrate many similar properties in binding to actin, tropomyosin, and calcium binding proteins [Takahashi *et al.* (1986), (1988), Sobue *et al.* (1981), Graceffa (1987)], and in inhibition of actomyosin ATPase activity [Ngai & Walsh (1984), Winder & Walsh (1990)].

CALPONIN

An additional 34 kDa component of smooth muscle thin filaments was first described by Takahashi *et al.* in 1986 from the heat soluble fraction of avian smooth muscle. Calponin was described as having a Stokes radius of 27 Å by gel filtration and by chromatofocusing to have a major band at pH 8.2-8.3 and a minor band at pH 7.9-8.0. Two isoforms of chicken gizzard calponin have been sequenced (Takahashi & Nadal-Ginard, 1991), a major isoform of 292 amino acids (figures 1.5, 1.6), and a minor isoform of 252 residues, the difference of 40 amino acids represents the deletion of a repeating unit in the smaller protein. Like caldesmon, it was shown to interact with F-actin, tropomyosin, and F-actin-tropomyosin in a calcium independent manner, and with calmodulin in a calcium dependent manner. Calponin also bound to a TNC affinity column, but not to a

MET	SER	ASN	ALA	ASN	PHE	ASN	ARG	GLY	PRO	10
ALA	TYR	GLY	LEU	SER	ALA	GLU	VAL	LYS	ASN	20
LYS	LEU	ALA	GLN	LYS	TYR	ASP	PRO	GLN	THR	30
GLU	ARG	GLN	LEU	ARG	VAL	TRP	ILE	GLU	GLY	40
ALA	THR	GLY	ARG	ARG	ILE	GLY	ASP	ASN	PHE	50
MET	ASP	GLY	LEU	LYS	ASP	GLY	VAL	ILE	LEU	60
CYS	GLU	LEU	ILE	ASN	LYS	LEU	GLN	PRO	GLY	70
SER	VAL	GLN	LYS	VAL	ASN	ASP	PRO	VAL	GLN	80
ASN	TRP	HIS	LYS	LEU	GLU	ASN	ILE	GLY	ASN	90
PHE	LEU	ARG	ALA	ILE	LYS	HIS	TYR	GLY	VAL	100
LYS	PRO	HIS	ASP	ILE	PHE	GLU	ALA	ASN	ASP	110
LEU	PHE	GLU	ASN	THR	ASN	HIS	THR	GLN	VAL	120
GLN	SER	THR	LEU	ILE	ALA	LEU	ALA	SER	GLN	130
ALA	LYS	THR	LYS	GLY	ASN	ASN	VAL	GLY	LEU	140
GLY	VAL	LYS	TYR	ALA	GLU	LYS	GLN	GLN	ARG	150
ARG	PHE	GLN	PRO	GLU	LYS	LEU	ARG	GLU	GLY	160
ARG	ASN	ILE	ILE	GLY	LEU	GLN	MET	GLY	THR	170
ASN	LYS	PHE	ALA	SER	GLN	GLN	GLY	MET	THR	180
ALA	TYR	GLY	THR	ARG	ARG	HIS	LEU	TYR	ASP	190
PRO	LYS	LEU	GLY	THR	ASP	GLN	PRO	LEU	ASP	200
GLN	ALA	THR	ILE	SER	LEU	GLN	MET	GLY	THR	210
ASN	LYS	GLY	ALA	SER	GLN	ALA	GLY	MET	THR	220
ALA	PRO	GLY	THR	LYS	ARG	GLN	ILE	PHE	GLU	230
PRO	SER	LEU	GLY	MET	GLU	ARG	CYS	ASP	THR	240
ASN	ILE	ILE	GLY	LEU	GLN	MET	GLY	SER	ASN	250
LYS	GLY	ALA	SER	GLN	GLN	GLY	MET	THR	VAL	260
TYR	GLY	LEU	PRO	ARG	GLN	VAL	TYR	ASP	PRO	270
LYS	TYR	CYS	ASP	ALA	PRO	GLY	LEU	LEU	GLY	280
GLU	ASP	GLY	LEU	ASN	HIS	SER	PHE	TYR	ASN	290
SER	GLN									292

Figure 1.5: Amino acid sequence of chicken gizzard calponin according to Takahashi & Nadal-Ginard (1991).

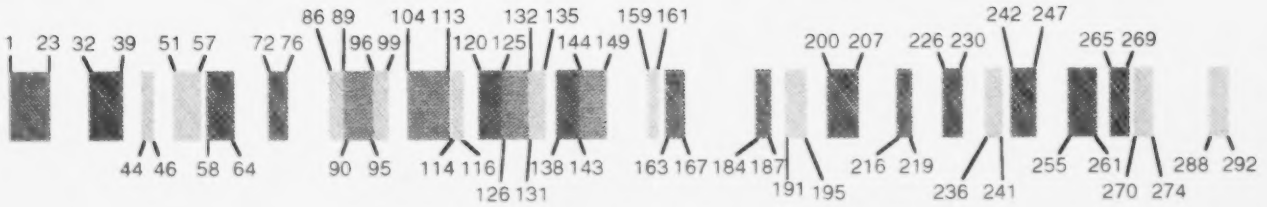

Figure 1.6: Predicted α-helical (green), β-sheet (brown), and β-turn (gold) structure of calponin based upon the amino acid sequence of Takahashi & Nadal-Ginard (1991), and the prediction method of Garnier *et al.* (1978).

TNI column (Takahashi *et al.*, 1988a). TNC binding in the absence of calcium was observed below 100mM NaCl, but became calcium dependent above 100 mM NaCl. Calponin shares a common antigenic determinant with the carboxyl terminal segment of rabbit skeletal and bovine cardiac TNT, and Takahashi suggested calponin may be a TNT like protein of smooth muscle (Takahashi *et al.*, 1988b). Later it was shown by Winder & Walsh (1990) that calponin was able to inhibit the actomyosin ATPase, suggesting that calponin was a prime candidate for a smooth muscle regulatory protein.

These properties, together with calponin's location on the thin filament (Takahashi *et al.*, 1988a) identified it as a potential regulator of smooth muscle contraction. Winder and Walsh (1990) have shown calponin can inhibit actin activated Mg^{2+}ATPase of myosin by 78% at a ratio of one calponin per three actin monomers, and that this inhibition is independent of calcium. Abe *et al.* (1990) also showed calponin inhibited ATPase without affecting phosphorylation of myosin light chain. The inhibition itself was calcium independent and has been shown to be reversible both by phosphorylation of calponin (Winder & Walsh, 1990b) or by addition of Ca^{2+}/calmodulin [Abe *et al.* (1990), Makuch *et al.* (1991)]. It is well documented that caldesmon binds myosin (Hemric & Chalovich, 1990), and it has recently been proposed that calponin also binds myosin [Lin *et al.* (1993), Szymanski & Tao (1993)], although the conditions may not be relevant in that the interaction does not occur at physiological salt concentrations. Both proteins can inhibit actin mobility on immobilized myosin, although their mechanism of action

appears to differ in that calponin behaves in an all or none fashion, while caldesmon produces a graded response (Shirinsky *et al.*, 1992). The relative importance and function of these two proteins is yet to be worked out. Makuch *et al.*, (1991) studied the interaction of calponin and caldesmon. They found that when F-actin complexed with calponin was subjected to increasing amounts of caldesmon, the caldesmon was able to compete off the calponin, and similarly caldesmon bound to F-actin was able to be displaced by increasing amounts of calponin. Since caldesmon and calponin apparently compete for actin binding sites on the thin filament and apparently do not form a complex with each other, they are not analogous to the troponin subunits of skeletal muscle. Tropomyosin does not seem to influence calponin's inhibition of the actin activated myosin ATPase [(Makuch *et al.* (1991), Winder & Walsh (1990a)], and its role in calponin function and in calponin binding on thin filaments remains undefined. It has been documented that as contraction progresses there is a loss of myosin light chain phosphorylation while the force of contraction continues [reviewed in Hai & Murphy (1989)], and an important objective of the research on calponin is to elucidate the mechanism for this behavior.

TISSUE DISTRIBUTION

Distribution of the immunoreactive components of calponin, tested by immunoblot analysis in bovine tissue by rabbit polyclonal antibodies, showed that

proteins of molecular weight 33 to 35 kDa that cross reacted with calponin antibodies were detected in all smooth muscle including aorta, esophagus, stomach, trachea, and uterus (Takahashi *et al.*, 1987). In addition non-smooth muscle tissue of the adrenal medulla and cortex showed cross reactivity (Takahashi *et al.*, 1987) as well as a study which localized proteins which reacted with calponin antibodies to platelets and fibroblasts (Takeuchi *et al.*, 1991). The findings of Takahashi *et al.* were later confirmed by Gimona *et al.* (1990) who were unable to detect calponin in cardiac, skeletal, kidney, spleen, or liver tissues. In addition, a number of detailed studies have been undertaken to localize calponin within the smooth muscle tissue. There has been some controversy over the presence of calponin in thin filaments (Lehman, 1989), but it has been shown that calponin's presence in the thin filament is dependent upon the preparation procedure being ATP free, which was not the case for the researchers who could not find calponin in the thin filament preparations (Nishida *et al.*, 1990).

CELLULAR LOCALIZATION

Electron microscopy supports the idea that calponin is a bona fide thin filament protein: electron microscopy of smooth muscle tropomyosin paracrystals indicated calponin binds to a site 16-17 nm from the carboxyl terminus of tropomyosin with a 40 nm periodicity, ie. identical to TNT (Takahashi *et al.*, 1988b). Further evidence that calponin is a component of thin filaments comes from the

fact that the thin filament bound form of calponin is degraded by calpain 500 times more slowly than the free form which indicates a close association with the thin filament proteins (Tsunekawa *et al*., 1989). Calponin is also degraded faster when bound to calmodulin indicating this complex formation leads to some structural change in the calponin molecule.

Several groups have monitored the expression of calponin in culture in order to determine if it can be used as a marker of smooth muscle differentiation. Indeed it has been found by Gimona *et al*. (1990) using monoclonal antibodies for avian calponin, that upon cultivation of embryonic chicken gizzards there was down regulation of expression. Calponin was found in stress fibres initially, then disappeared from the cells. This loss of calponin accompanied loss of metavinculin and the change of caldesmon isoforms from the high to the low molecular weight variant. Biryukov *et al.* (1991), also using polyclonal antibodies to chicken gizzard calponin, examined rabbit and human aortic smooth muscle cells in culture. They localized calponin expression to microfilament bundles, and found that with time calponin disappeared from some cells, while others localized calponin to the stress fibres. They felt calponin was a marker of the contractile phenotype, since with culture expression decreases ~9x to the level of fibroblasts, and endothelial cells, these latter two were found to contain low amounts of calponin. The fact that calponin expression disappears as the muscle cell dedifferentiates supports the idea that calponin plays a role in contraction.

There is also evidence of localization of calponin to the cytoskeleton. The

work of Takeuchi *et al.* (1991) on bovine platelets and mouse fibroblasts localized calponin to stress fibres, actin filaments, and dissociated from actin filaments which led them to propose calponin may be involved in the dynamic regulation of cytoskeletal structure. Lehman (1991) found he was able to isolate caldesmon containing thin filaments virtually devoid of calponin, and filamin and calponin containing filaments with no caldesmon present. This led him to postulate there were distinct types of thin filaments *in vivo*. This had already been suggested by the work of Furst *et al.* (1986) and Small *et al.* (1986) who showed using immunocytochemical studies that there are at least two classes of thin filaments that exist in smooth muscle, one containing caldesmon and localized with myosin in a contractile-actomyosin domain, and another containing filamin and localized in a cytoskeletal actin intermediate filament domain. North *et al.* (1994) localized calponin to the cytoplasm with β-cytoplasmic actin, filamin, and desmin, as well as in the contractile apparatus with myosin and caldesmon, however it was much more concentrated in cytoskeleton. While some groups suggest calponin is predominantly in the cytoskeleton, some in the stress fibres of cells in culture, and some simply that calponin colocalizes with actin and tropomyosin (Walsh *et al.*, 1993), all studies agree calponin colocalizes with actin microfilaments.

PHOSPHORYLATION

In order to determine if phosphorylation might play a role in calponin

regulation, Winder & Walsh (1990a) demonstrated that calponin could be phosphorylated both by protein kinase C, and by calcium/calmodulin dependent protein kinase II, but not by cAMP or cGMP protein kinases or by MLCK. This phosphorylation was shown to affect calponin binding to actin by cosedimentation studies, but not calmodulin or tropomyosin binding by affinity chromatography. This study went on to show that phosphorylation of calponin abolished the ability of this protein to inhibit the actin activated myosin ATPase without affecting myosin light chain phosphorylation establishing phosphorylation as a putative regulatory mechanism. The ability of PKC to phosphorylate calponin has been confirmed by the work of Naka *et al.* (1990), and Nakamura *et al.* (1993). Work has been done to identify the sites of phosphorylation. Winder *et al.* (1993) have identified Ser 175 and Ser 234 as the major sites of phosphorylation, with minor sites at Ser 215, Thr 180, Thr 184, and Thr 259. Using calcium/calmodulin dependent protein kinase II they found Ser 175 as the major site with some phosphorylation at Ser 71, 254, and 215. In contrast Nakamura *et al.* (1993) have identified Thr 184 as the major site of phosphorlation with PKC, with minor phosphorylation occuring at Ser 215, Thr 220, Thr 224, and Thr 259. While the exact site of phosphorylation appears to be open from these two studies, in both cases the fragment 173-185 has been identified from tryptic digest and identification of phosphorlated peptides as the most important phosphorylated segment. The study of Nakamura *et al.* (1993) went on to show actin was a competitive inhibitor of phosphorylation at this site, suggesting the phosphorylation site is close to the actin binding site. In this same

study it was found that tropomyosin was an uncompetitive inhibitor. In a previous study by Naka *et al.* (1990), calmodulin was shown to be a non competitive inhibitor of phosphorylation. From thes findings, the interpretation was made that the phosphorylation site is not near the tropomyosin or calmodulin binding sites, and the calmodulin, tropomyosin, and actin binding sites are all separate since they affect phosphorylation in different ways. Nakamura *et al.* (1993) also looked at the effect of calponin phosphorylation upon the binding to actin, tropomyosin, and calmodulin. They were able to confirm, through cosedimentation studies, the earlier results of Winder & Walsh (1990a) that phosphorylation diminished the ability to bind to actin; however through the use of dansyl labelled calmodulin and tropomyosin, they were also able to demonstrate effects of calponin phosphorylation upon the interaction with these two proteins. Tropomyosin exhibited lower affinity for phospho-calponin, while for calmodulin there was no effect upon binding affinity; however phosphorylation does modulate the interaction, because the magnitude of the fluorescence change upon interaction was greatly reduced suggesting an effect on the ability of calponin to cause a structural change in calmodulin. These workers also showed that there was not any endogenous phosphate in purified chicken gizzard calponin, and that tropomyosin had no effect upon the affinity of calponin or phospho-calponin for actin.

PHOSPHATASES

Two groups have also identified tentative phosphatases isolated from smooth muscle which are capable of dephosphorylating *in vitro* PKC phosphorylated calponin, and restore its inhibition abilities. Winder *et al.* (1992) isolated smooth muscle phosphatase I, which is known to dephosphorylate isolated myosin light chain, as the active phosphatase acting on calponin. This is classified as a type 2a protein phosphatase. Ichikawa *et al.* (1993) isolated myosin binding phosphatase which is a 1δ protein phosphatase, and demonstrated that calponin is a very good substrate for this phosphatase also, making the argument that the presence of a phosphatase capable of restoring calponin's inhibitory activity, establishes both an on (phosphorylation) and an off (phosphatase) switch for calponin.

IN VIVO PHOSPHORYLATION

In order to determine if phosphorylation is a mechanism of calponin regulation *in vivo*, a number of groups have undertaken to establish, using radioactive phosphate, if calponin incorporates phosphate during the contraction of muscle. This work has become the subject of great controversy. Pohl *et al.* showed in 1991 that canine tracheal smooth muscle strips stimulated by carbachol

did incorporate phosphate in calponin during contraction, while Bárány *et al.* (1991) found that there was not incorporation of phosphate into pig carotid artery using KCl, norepinephrine, histamine or PDBU as stimulants, and Gimona *et al.* (1992) showed there was no phosphate incorporation into carbachol stimulated chicken gizzard or guinea pig taenia coli. This suggested the sites available for phosphorylation *in vitro* may not be available *in vivo*, or the appropriate conditions for phosphorylation are not being met. Since then, additional studies have come out for [Winder *et al.* (1993), Pohl & Gerthoffer (1994), Rokolya & Moreland (1994)] and against [Bárány & Bárány (1993), Adam *et al.* (1994)] *in vivo* phosphorylation of calponin, leaving this question as yet unanswered.

MECHANISM OF ACTION

The regulation of calponin function is being characterized *in vitro* using solution biochemistry and motility assays. Studies on the enzyme kinetics of acto-HMM in the presence of calponin determined that calponin lowered the K_{ATPase} by less than 2 fold, however the V_{max} of the reaction was lowered 6 fold (Horiuchi & Chacko, 1991). This suggests that calponin affects the catalytic step in the ATPase, with only a small effect on the binding of HMM to actin. They also note that the tropomyosin effect is similar to that seen by the cooperative turning on of thin filaments in the presence of slowly cycling crossbridges, and suggests calponin may be causing these slowly cycling crossbridges.

It has been shown that calponin is able to inhibit the covalently crosslinked complex of actin with S1, which suggests that calponin does not sterically inhibit this interaction in order to inhibit ATPase, but rather may play a role in altering actin's structure such that a catalytic step is modified (Miki *et al.*, 1992). This work also showed that modification of Lys 61 of actin with FITC, which interferes with the inhibitory behavior of troponin-tropomyosin, did not interfere with the ability of calponin to inhibit acto-HMM ATPase. Furthermore, modification of the amino terminal of actin by covalently attaching AEANA was unable to interfere with the binding of actin to calponin, which is unlike the situation with caldesmon. This suggests calponin acts by a mechanism unique from either troponin or caldesmon in inhibiting the acto-myosin ATPase. Shirinsky *et al*. (1992) used *in vitro* motility assays to show that calponin inhibited movement of actin in an all or none fashion. This work was in line with the results of Horiuchi and Chacko which indicated calponin did not inhibit binding of actin to myosin in the solution ATPase assay since calponin affected V_{max} but not K_{ATPase}, implying that calponin inhibits a catalytic step, not direct steric hinderance of myosin binding to actin. This mode of action would not affect the number of attached crossbridges, but rather would affect shortening velocity. Haeberle (1994) found that calponin in *in vitro* motility assays, stopped velocity while increasing force, and simultaneously increased actin binding to myosin, suggesting calponin may be stabilizing the strong actin myosin interaction and slowing crossbridge cycling. This behavior suggests calponin is a prime candidate for the load bearing interaction in a latch bridge state.

The role of tropomyosin in calponin regulation is still being elucidated. Winder & Walsh (1990a) found no effect of tropomyosin on their solution ATPase assays, a result confirmed by the *in vitro* motility assays of Shirinsky *et al.*. However, Horiuchi & Chacko found tropomyosin-actin was inhibited only 2.5 fold by calponin vs. 6 fold inhibition of actin alone.

INTERACTION OF CALPONIN WITH CALCIUM BINDING PROTEINS

Two potential roles for calponin, as a component of the thin filament, have been identified in smooth muscle contraction, either as a regulator of the actin myosin interaction as described above, or as a component of the cytoskeleton, potentially serving to regulate arrangement of the actin filaments [Takeuchi *et al.* (1991), North *et al.* (1994)]. Either of these mechanisms would work in concert with the phosphorylation of myosin light chain, which has been identified as a central regulatory event in smooth muscle contraction [Adelstein & Eisenberg (1980), Hartshorne (1987)].

Calponin, when bound to actin, serves to inhibit actin's interaction with myosin, and may well inhibit actin's interaction with cytoskeletal components such as filamin. In order for contraction to occur there must be a release of this inhibition. It has been shown that calponin can be phosphorylated *in vitro*, and that phosphorylation prevents calponin's inhibitory activity (Winder & Walsh, 1990a). There is controversy over whether calponin is phosphorylated *in vivo*, and

the biological relevance of this regulatory mechanism is yet to be established [Gimona *et al.* (1992), Winder *et al.* (1993), Bárány & Bárány (1993)]. A second putative regulatory mechanism that has been suggested is modulation of calponin's inhibitory activity through interaction with calcium binding proteins which releases calponin's inhibition of actin activated myosin ATPase. Based upon the ability of calmodulin to modulate calponin's function, thesis research was undertaken in the first instance to characterize the calponin-calmodulin interaction and determine its biological relevance. These studies explored whether calmodulin was the most appropriate candidate for regulation of calponin through a physical characterization of the strength and nature of the interaction.

In order to determine whether the complex formed between calponin and calmodulin was specific, or merely the result of the highly basic nature of calponin interacting with the acidic nature of calmodulin, it was undertaken to examine the interaction of a number of calcium binding proteins with calponin. It was found that not only did calponin interact with a number of calcium binding proteins which exposed hydrophobic patches upon binding calcium, but in some cases the strength of interaction was stronger than with calmodulin. This was the case with S-100b and the protein caltropin. Since caltropin is isolated from avian gizzards, it was a logical choice to examine as a regulatory protein for calponin. We were able to demonstrate that caltropin could interact and regulate calponin with greater efficiency than calmodulin could.

During the course of investigating the relationship between calponin and the

calcium binding proteins, we were able to determine that the stoichiometry of interaction between these two proteins is 2 mols of calcium binding protein to 1 mol of calponin. It was of interest, therefore, to further localize the two binding sites of calcium binding proteins upon calponin. Chemical cleavage of calponin provided us with the amino terminal portion of the protein which we found was able to interact with calcium binding protein. By means of a collaboration, we were able to obtain deletion mutants of calponin through which we localized the second interaction site near the actin binding region in the centre of the molecule. This thesis work was the first physico-chemical analysis of the calponin - calcium binding protein interaction, and the correlation of this data with the biological results will contribute to determining the role of calponin in smooth muscle contraction.

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Chapter 2

EXPERIMENTAL PROCEDURES

PROTEIN PURIFICATIONS

Calponin was purified from turkey gizzards by a modification of the method of Takahashi *et al.* (1986, 1988) as described in chapter 3. Bovine brain calmodulin, rabbit skeletal troponin C, rabbit skeletal myosin and actin, and pike parvalbumin were prepared by routine procedures used in this laboratory (Cachia *et al.*, 1986; M^{c}Cubbin *et al.*, 1982; Margossian and Lowey, 1982; Pardee and Spudich, 1982; Henryl *et al.*, 1985, respectively). Bovine brain S-100b was prepared using the procedure for purifying the α-chain of S-100a (Leung *et al.*, 1986). Peak IV obained from the DEAE-Sephadex A-25 column was subjected to reversed phase HPLC on a C_{18} column employing a solvent of 0.1% TFA with a 1%/min acetonitrile gradient to yield S-100b. Chicken gizzard SMCaBP-11 protein was prepared as described by Mani and Kay (1990). Final purification and removal of metal ions from calmodulin and troponin C was also achieved using the reversed phase HPLC according to the procedure of Ingraham and Hodges (1988).

For the chapters on the interaction of calponin and its fragments with caltropin, caltropin was isolated from turkey gizzards according to the method of Mani & Kay (1990). For the interaction work with the calponin fragments, calmodulin was isolated from turkey gizzards as a by product of the caltropin purification in which peak IV off the Q-Sepharose column is calmodulin (Mani &

Kay, 1990) which was then subjected to RP HPLC from which it elutes at ~ 44% acetonitrile. The fragment comprising CP 2-51 was isolated from a CNBr digest of turkey gizzard calponin in 70% formic acid. The digest was lyophilized, then taken up in 10 mM MOPS pH 7.0, 1 mM $CaCl_2$, 1 mM DTT, and 0.01% NaN_3 and applied to a calmodulin affinity column. The fragment eluted when the column was washed with 2 mM EGTA. This pool was then applied to a C18 reversed phase HPLC column from which it eluted at 30% acetonitrile. The identity of the fragment was confirmed by amino acid compositional analysis, and sequencing.

The calponin mutants were provided by Dr. Mario Gimona and Dr. Peter Strasser at the Institute for Molecular Biology in Salzburg, Austria. The protocol as provided by these collaborators is as follows. The expression system employed for producing the recombinant mouse calponin fragments 1-228 and 45-228 used in this study was as described previously (Strasser *et al.*, 1993). As there are differences in sequence between avian and mouse calponin, we used porcine calponin as a control because it has a very similar sequence to mouse calponin (Strasser *et al.*, 1993), and was more readily available than full length mouse calponin. Secondary structure analysis controls were run between avian and porcine calponin to demonstrate they had similar properties. The calponin constructs were designed using existing restriction sites in the cDNA. To maintain the correct reading frame the ends of the restriction fragments had to be modified in some cases prior to subcloning into the expression vector (blunt-ending by Mung bean nuclease, filling in by Klenow fragment, inserting of linkers). All

products were checked by sequencing. In order to purify these fragments, bacterial cells were centrifuged at 5000 rpm and the pellet dissolved in 5 mM KH_2PO_4, 5 mM K_2HPO_4, 10 mM NaCl, 2 mM EDTA, 2 mM EGTA, pH 7.0. A french press was used to open the cells and the suspension clarified by centrifugation at 18 000 rpm. Ammonium sulphate cuts from 0-30 and 30-60% saturation were performed. The 30-60% pellet was redissolved in the same phosphate buffer as above and applied onto a hydroxylapatite column (2.5 x 10 cm) equilibrated in the same buffer. A linear phosphate gradient (2x150 ml, 10-250 mM PO_4) was used to elute the recombinant protein which eluted at ~180 mM. The peak fractions were pooled and precipitated with ammonium sulphate at 60 % saturation. The pellet was dissolved in 20 mM MES, 10 mM NaCl, 2 mM EGTA, 2 mM EDTA, pH 5.4 and loaded onto an S-Sepharose column equilibrated in the same buffer. Bound protein was eluted in a single step by applying 1 M NaCl in the same buffer and the peak fractions were collected. After concentration by ammonium sulphate precipitation the pellets were dissolved in 50 mM MOPS pH 7.1, 100 mM NaCl, 1 mM DTT and purified on an FPLC Superose 12 column (1x30 cm). The 45-228 fragment was not soluble after the french press treatment so 4 M urea was added to the crude french press extract and the hydroxylapatite column was run in 4 M urea from which the protein eluted at ~20-40 mM PO_4. 4 M urea was also used in the phosphate buffer to purify this fragment on a S-100 Sepharose fast flow column and then this fragment was purified on the FPLC column as described above.

REVERSED PHASE LIQUID CHROMATOGRAPHY

Reversed phase high performance liquid chromatography was performed on a Varian Vista 5000 Liquid Chromatography system equipped with a Vista 402 Data System. Unless otherwise stated, a SynChropak RP-P (C18) 250 x 10 mm column was employed, and the protein was dissolved in 0.1% TFA prior to injection onto the column. The sample was eluted with a linear AB gradient, where A = 0.1% TFA in water, and B = 0.05% TFA, 70% acetonitrile.

POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was used for molecular weight determination, and estimation of purity for protein samples. Electrophoresis was performed in the presence of SDS using the buffer system of Laemmli (1970). A Biorad Minigel apparatus was employed with a final acrylamide concentration of 15%, and 0.4% bisacrylamide. Prior to being applied to the gel, protein samples were dissolved in 5% β-mercaptoethanol, 2% SDS, and 0.125% bromophenol blue as a dye front marker. The sample was placed in boiling water for 2 minutes in order to equilibrate with the sample buffer. The gel was run at a constant voltage of 200 mV for approximately 45 minutes using the low molecular weight markers provided by Biorad as standards. Following electrophoresis, the gel was washed with 10%

acetic acid, 45% methanol for 20 minutes, then stained with the Fast Stain method (Zoion Research Inc., Newton, MA) for 30 minutes, and finally destained for several hours in 10% acetic acid.

AMINO ACID ANALYSIS

Amino acid analysis of the two isoforms of calponin was performed on a Dionex D500 amino acid analyzer. Detection was accomplished by post column ninhydrin. The sample was hydrolyzed in 6 HCl @ 100°C for 24 hrs without extended time course studies. For cysteine determination, 2% DMSO was included in the hydrolysis mixture as described by Spencer and Wold (1969). Tryptophan, methionine, threonine, and serine were determined by hydrolysis in methanesulphonic acid @160 °C for 1 hr according to the procedure of Chiou and Wang (1988). The results represent the average of at least three preparations for each method. All other protein samples were hydrolyzed for 1 hr @ 160°C in 6N HCl/0.1% phenol. The samples were analyzed on a Beckman 6300 ion exchange HPLC amino acid analyzer. Amino acids were detected by post column ninhydrin.

PROTEIN SEQUENCING

Sequencing was performed on an Applied Biosystems 473A pulsed liquid/gas-phase protein sequencer. The sample was loaded on a precycled

polybrene-coated glass fiber disc and subjected to standard Edman degradation chemistry.

MASS SPECTROSCOPY OF CALPONIN

Mass spectrometric analysis of calponin was performed on a VG Biotech (Fisons Instruments) mass spectrometer by platform electrospray ionization.

PROTEIN CONCENTRATIONS

Proteins concentrations were established in a Beckman Spinco Model E Ultracentrifuge using the Raleigh optical system at 8000 rpm and 20°C. An extinction coefficient for calponin was determined by comparing the magnitude of the absorption peak observed at 276nm with the results of an analytical ultracentrifuge experiment on the same sample to determine a fringe count. The relationship 4.1 fringes = 1mg/ml protein solution was used (Babul and Stellwagen 1969). This concentration was confirmed by amino acid analysis of the sample using the amino acid composition of Takahashi & Nadal-Ginard (1991) for the 32kDa isoform. An $A^{1\%}_{276nm}$ of 7.5 was determined and this value represents an average of five separate sample. This extinction coefficient was used to determine protein concentration for experiments and was routinely confirmed by amino acid analysis.

The following extinction coefficients were used to determine protein concentrations: calmodulin $A^{1\%}_{277nm}$ = 1.95 (Klee, 1977), TNC $A^{1\%}_{277nm}$ = 1.75 (M^{c}Cubbin *et al.*, 1982), parvalbumin $A^{1\%}_{259nm}$ = 1.54 (Henryl *et al.*, 1985), S-100b $A^{1\%}_{278nm}$ = 2.4 (Mani *et al.*, 1982), myosin $A^{1\%}_{280nm}$ = 5.88 (Verpoorte & Kay, 1966), and actin $A^{1\%}_{290nm}$ = 6.3 (Lehrer & Kerwar, 1972), and S1, $A^{1\%}_{280nm}$ =8.1 (Margossian & Lowey, 1982). Absorption spectroscopy was performed on a Perkin Elmer Lambda 5 spectrophotometer. S-100b and caltropin concentrations were determined from amino acid analysis from the compositions of Marshak *et al.* (1981), and Mani & Kay (1990) respectively.

The following extinction coefficients used were calculated from the sequences using $E^{1M}_{279.8nm}$ =5600 for tryptophan, and $E^{1M}_{279.8nm}$ =1420 for tyrosine (Gratzer, 1989): CP 1-228, $A^{1\%}_{276nm}$ =7.7; CP 45-228 $A^{1\%}_{276nm}$ =5.16; CP 2-51 $A^{1\%}_{276nm}$ =15.92; porcine calponin $A^{1\%}_{276nm}$ =8.9.

Acrylodan label concentration was determined by A^{1M}_{387nm} = 16400 (Prendergast 1983). The concentration of labelled protein was established by amino acid analysis using the composition for calponin of the α-isoform sequenced by Takahashi & Nadal-Ginard (1991).

GEL FILTRATION OF CALPONIN ISOFORMS

Stokes radius was evaluated by HPLC on a Varian 5000. A Pharmacia Superose 12 column (1x30 cm) was equilibrated in 25 mM Tris-HCl ph 7.5, 100

mM KCl, 1 mM DTT at 22°C and 1 ml/min. The standard curve was established by plotting: $(-\log K_{av})^{1/2}$ vs. Stokes radius where K_{av} = (Ve-Vo)/(Vt-Vo) as in Siegel and Monty (1966). The standards used were bovine serum albumin 35Å, ovalbumin 28.4Å, chymotrypsinogen 21.9Å, myoglobin 19.8Å, cytochrome c 17.2Å (Byers & Kay, 1982), and β-lactoglobulin 26.6Å (Tanford, 1967). Also a value of 22.5 Å was calculated for carbonic anydrase using a molecular weight of 29 500 Da and $S^{\circ}_{20,w}$=2.85S (Winzor, 1969). The theoretical Stokes radius for a globular protein of the same molecular weight as calponin was calculated according to Byers & Kay (1982).

ABSORPTION SPECTROSCOPY

Absorption and UV difference spectroscopy were performed on a Perkin Elmer Lambda 5 spectrophotometer. UV difference spectroscopy for detecting Ca^{2+} binding was performed over the wavelength range 250 to 350 nm in 1 cm path length cells. With 18 μM protein solutions in both cells, additions from 0.1μM to 2 mM free Ca^{2+} were added to the sample cell while equal volumes of H_2O were added to the blank cell, and the spectrum was then scanned to detect any changes. The buffer was 25 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA.

CIRCULAR DICHROISM SPECTROSCOPY

Circular dichroism spectra arise from the differential absorption of left and right circularly polarized light, and is defined as $\theta = 2.303(A_L - A_R)180/4\pi = 33\Delta A$ degrees, where A_L and A_R are the absorption of left and right circularly polarized light, respectively in degrees. The absorption is expressed in terms of molar ellipticity $[\theta]$, defined by the equation $[\theta] = \frac{mrw \cdot \theta_{obs}}{10 \cdot d \cdot c}$, where mrw is the mean residue weight calculated from the amino acid composition, θ is the observed ellipticity, d is the pathlength of the cell in decimeters, and c is the concentration of the sample in g/ml. The units of $[\theta]$ are degrees cm^2 $dmol^{-1}$.

Circular dichroism measurements were performed on a Jasco J-720 spectropolarimeter. The cell was maintained at 25°C with an RMS circulating water bath (Lauda, Westbury, N.Y.). Far UV scans from 255 nm to 195 nm employed a cell with path length of 0.01 or 0.02 cm. The computer averaged 10 scans and the signal due to solvent was subtracted. The instrument was routinely calibrated with d(+)-10-camphorsulfonic acid at 290 nm and with pantoyl lactone at 219 nm by following procedures outlined by the manufacturer. The data were plotted as mean residue weight ellipticity (expressed in degrees square centimeters per decimole) vs. wavelength in nanometers. Spectra for the calponin isoforms were determined in 25 mm Tris-HCl pH 7.5, 100 mM NaCl, and 1 mM DTT. The protein concentration was ~ 1 mg/ml for far UV measurements, ~0.5 mg/ml for near UV,

while the mean residue ellipticity, [θ] was calculated using a mean residue weight of 110.9 for calponin and expressed in degree cm^2/dmol. The experiments with acrylodan labelled calcium binding proteins and calponin used a total of ~ 1mg/ml in the cell in a buffer of 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA. For the experiments with labelled calponin, the concentration of calponin and acrylodan calponin was 30μM, while 10μM calponin was used in the complexation experiment with a 1:1 mol ratio of calmodulin. The experiments involving fragments of calponin were performed in 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA with and without 3 mM $CaCl_2$, and the protein concentrations were in the range of 0.3 - 0.9 mg/ml. The mean residue weight of calponin used was 110.7 Da/residue calculated from the sequence published by Takahashi & Nadal-Ginard (1991). The pH profile of calponin was determined by monitoring the ellipticity at 221 nm while aliquots of HCl or NaOH were added to the sample in order to achieve the desired pH. The solvent used was 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA with and without 3 mM $CaCl_2$. The protein concentration was 0.562 mg/ml in a 0.05 cm cell. Theoretical curves were generated by adding the contributions of the constituent proteins in the ratios of their relative mass present during the experiment. The guanidine hydrochloride denaturation profile was monitored at 221 nm using a total protein concentration in the cell of 0.5 mg/ml. All of the samples were made up separately and allowed to equilibrate overnight before measurements were taken. Estimations of ΔG_D and $\Delta G_D^{H_2O}$ where $\Delta G_D^{H_2O}$ is the free energy of

unfolding in H_20 were calculated according to Pace (1986). The temperature denaturation was also followed at 221 nm using a total protein concentration in the cell of 0.5 mg/ml. ΔH values were calculated from Van't Hoff plots employing the relation $\Delta H = -R(d \ln K)/d(1/T)$ where R is the gas constant, T is the temperature in °K, ΔH is the enthalpy difference, (M°Cubbin *et al.*, 1980), and the equilibrium constant K was calculated according to Pace (1975).

ANALYTICAL ULTRACENTRIFUGATION

Ultracentrifugation studies were performed on a Beckman Spinco model E analytical ultracentifuge. Molecular weights were determined by low speed sedimentation equilibrium employing Raleigh interference optics according to Chervenka (1969). The apparent weight average molecular weight, M_w of a sample is determined from the equation $M_w = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \cdot \frac{\delta \ln c(r)}{\delta r^2}$ where R is the universal gas constant, T is the temperature in °K, $\bar{v}$ is the partial specific volume of the protein, ρ is the solvent density, ω is the angular velocity, and c is the protein concentration at distance r from the axis of rotation. Protein molecular weights were determined by plotting the natural logarithm of the protein concentration versus r^2, the slope of which is $\frac{\delta \ln y}{\delta r^2}$ and was used to determine the molecular weight at any position along the cell (Chervenka, 1969).

For the interaction with calmodulin, protein concentrations were in the range of 1-2 mg/ml and the molecular weights were calculated from the slope of the lny

vs. r^2 plot. In figure 4.3, the initial loading concentrations and speeds were: calponin, 1.1 mg/ml and 16 000 rpm; calmodulin, 1.91 mg/ml and 18 000 rpm; and the complex, 1.7 mg/ml and 11 000 rpm. The temperature was maintained at 5 °C. For the interaction with caltropin, proteins were loaded at initial concentrations of 0.63 - 0.86 mg/ml, in 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA or 2 mM $CaCl_2$. Molecular masses were calculated from the slope of the lny vs. r^2 plot. Proteins were combined in ratios of 2 mols calponin to 1 mol caltropin, and 2 mols caltropin to 1 mol calponin, in the presence and absence of Ca^{2+}, in order to determine the nature of the complex. For interaction studies with CP 1-228, proteins were loaded at an initial concentration of 0.6-0.8 mg/ml, in 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, and 3 mM $CaCl_2$.

FLUORESCENCE STUDIES

Fluorescence emission and excitation spectra were measured on a Perkin-Elmer MPF 44B recording spectrofluorimeter equipped with a DCSU-2 corrected spectra accessory which allows for automatic background fluorescence correction. The temperature was maintained at 20°C, fluorescence measurements were at 90° to the source, and slit widths were set at 5 nm. The instrument was operated in ratio mode. Absorption of the solution at the wavelength of excitation never exceeded 0.1, so no correction for the inner filter effect was necessary. All spectra

are corrected for solvent and for concentration changes. The fluorescence of acrylodan labelled calcium binding proteins was monitored at 460 nm, and the CaBP concentration was ~1.5 μM. For the scans of the complex, and for calcium titrations, the calponin was present in a 10 fold molar excess. Intrinsic tryptophan fluorescence of calponin was monitored by exciting at 295 nm and monitoring emission at 335 nm. Acrylodan calponin was excited at 388 nm and titrations with both Ca^{2+} and Ca^{2+} binding proteins were performed by monitoring at 460 nm - the wavelength of greatest change. The concentration of acrylodan calponin in the cell was .5 μM and the Ca^{2+} titration of calponin-calmodulin was performed with 10 mol ratios of calmodulin over calponin in order to saturate calponin. Overall effects were followed by scanning fluorescence emission from 400 to 600 nm. The buffer used was 50 mM MOPS pH 7.15, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA.

The fluorescence titrations of acrylodan calponin with calcium binding protein were performed in the presence of 3 mM $CaCl_2$. For the fluorescence scans the caltropin concentration was 2.0 μM, and $CaCl_2$ was added to a concentration of 3 mM. The Ca^{2+} titration was performed with 0.5 μM calponin, 1.0 μM caltropin dimer, and monitored at 460 nm.

ACRYLODAN LABELLING

6-Acryloyl-2-dimethylaminonaphthalene (acrylodan) is a fluorescent probe

which interacts covalently with free sulfhydryl groups (figure 2.1). Calponin was labelled with a 1.5 fold excess of acrylodan (Molecular Probes; Eugene, Oregon) over calponin in 50 mM MOPS pH 7.15, 100 mM NaCl, at a protein concentration of 1 mg/ml. The labelling was allowed to proceed overnight at 4°C, the reaction stopped by the addition of excess DTT, and the solution dialyzed to remove excess label. Wheat germ calmodulin was labelled according to Strasburg *et al.* (1988) and Mills *et al.*, 1988, troponin C according to Wang *et al.* (1987), and S-100b according to Mani & Kay (1985).

LOCATION OF ACRYLODAN LABEL

Determination of the location of the acrylodan probe on calponin was achieved by doing a complete tryptic digest of acrylodan labelled calponin in 50 mM Mops, pH 7.2, 100 mM NaCl, 1 mM DTT, and 8 M urea. 1/25 Trypsin (Sigma) to calponin (w/w) was added and the sample incubated @ 37°C for 24hr. The reaction was stopped by injecting onto a Zorbax 300SB C_8, 4.6 mm x25 cm RP HPLC column. A 1%/minute gradient was run over a range of 0-50% acetonitrile in 0.1% TFA, and a peak that eluted at 40% acetonitrile that had fluorescence characteristic of acrylodan in addition to protein absorption was collected. This peak was rerun at 0.5% per minute acetonitrile to further purify it, and then sequenced.

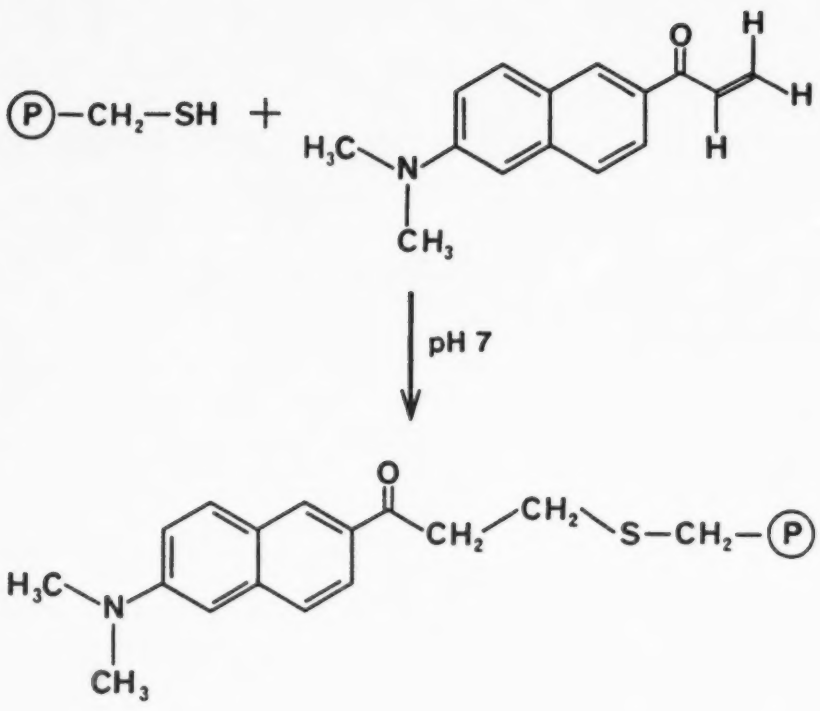
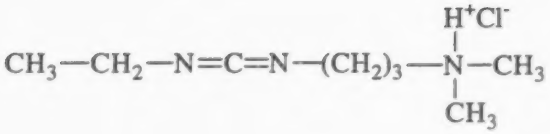


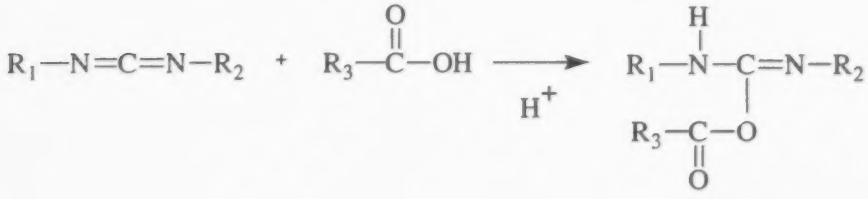
Figure 2.1: A general mechanism for the reaction of acrylodan with the exposed cysteine residues of proteins. This fluorescent probe, which interacts specifically with sulfydryl groups in a covalent manner, is highly sensitive to the polarity of its environment. This is attributed to the presence of a dimethylamino group at position 2 of the naphthalene ring which serves as an electron donor, and a C=O group at position 6 of the ring acting as an electron acceptor.

CROSSLINKING STUDIES

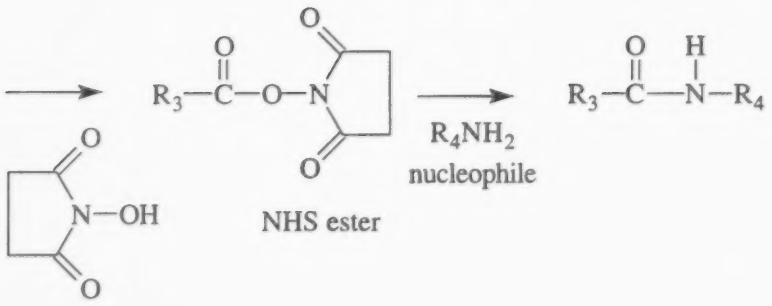
Crosslinking of calponin was performed in 50 mM MOPS pH 7.15, 100 mM NaCl, and 1.5 mM $CaCl_2$ in a volume of 1 ml. The calponin concentration was 4.3 uM, while the calmodulin concentration was 17.9 μM, a 4.2 fold excess. DTT was removed just prior to the reaction by running calponin over a 1 x 15 cm P6 desalting gel (Biorad). The crosslinking was started by adding 1-ethyl-3-[3(dimethylamino)propyl] carbodiimide (EDC) and N-hydroxysuccinimide [$NHS(SO_3)$] to a final concentration of 1 mM and aliquots were removed at 0, 1/2 hr, 1 hr, and 2 hrs. EDC reacts with carboxylic acid groups, activating these groups, and allowing them to be coupled to amino groups in the reaction mixture. The purpose of the $NHS(SO_3)$ is to form an active ester which is less subject to hydrolysis than the o-acyl urea intermediate of EDC (Means & Feeney, 1971). The reaction was stopped by adding SDS-PAGE sample buffer, of which the β-ME acts as a scavenger for excess reagent due to its nucleophile character. (figure 2.2) The samples were then run on SDS-PAGE in order to determine the molecular weight of the crosslinked species.



1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride



o-acyl urea



NHS ester

Figure 2.2: EDC forms an o-acyl urea with carboxyl groups, enabling the carboxyl group to then couple to N-hydroxysuccinimide. This ester subsequently reacts with amines resulting in the formation of a peptide bond.

QUIN 2 FLUORESCENCE TITRATIONS

Quin 2 is related to EGTA, with carboxyl groups able to chelate calcium (figure 2.3), and the fluorescence peak shifts from 510 to 525 nm with the intensity increasing 5 fold upon binding calcium (Thomas, 1982). Monitoring of Ca^{2+} binding with this fluorescent Ca^{2+} indicator was achieved by using the Amicon Centricon 10 ultracentrifugation device for rapid flow dialysis. Protein solutions were dialyzed in acid washed cylinders to reduce Ca^{2+} contamination, and in the presence of Chelex-100, in the buffer 25 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT. Known amounts of Ca^{2+} were added to 40 μM protein samples in the top chambers of prewashed centricons and centrifuged at 4200 rpm for 30 minutes to filter a portion of buffer through the 10 kDa cutoff membrane. Calponin which has a mass of 32 kDa was retained in the upper portion of the centricon. Aliquots of flow through buffer were added to a solution of 20 μM Quin 2 and 100 mM MOPS pH 7.2 to determine Ca^{2+} levels. The increase in fluorescence was monitored at an excitation wavelength of 339 nm and an emission wavelength of 492 nm. From the known amount of Ca^{2+} added, the determined free Ca^{2+} levels, and the amount of protein present, it is possible to calculate the amount of Ca^{2+} bound to the protein. The experiment was initially performed on TNC to ensure the method was accurate and on calponin to test for Ca^{2+} binding.

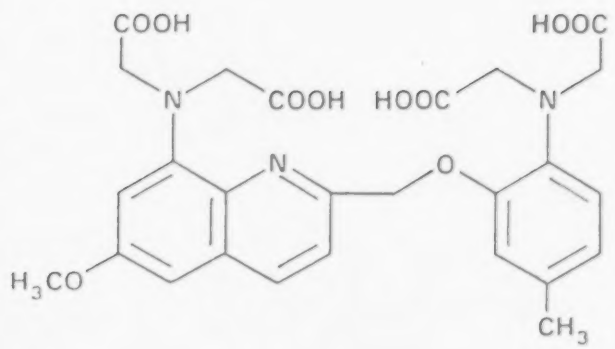


Figure 2.3: Structure of the fluorescent probe Quin 2. The tetraacetic acid moiety chelates calcium which increases the quantum yield of the molecule.

EQUILIBRIUM DIALYSIS

Equilibrium dialysis was performed in 50 ml centrifuge tubes. A 150 μl protein sample @ 1.3 mg/ml calponin was placed in 10-12 kDa molecular weight cut off dialysis tubing and dialyzed vs. 10 mM MOPS pH 7.0, 150 mM KCl, 100 μM EGTA, 2 mM DTT, .26 μM $^{45}Ca^{2+}$, and 200, 300, or 600 μM cold Ca^{2+} in triplicate. Samples were placed on a shaker and left at 4°C overnight. The free Ca^{2+} concentration was determined by the concentration of label in a 20 μl sample of dialysis buffer. The bound Ca^{2+} concentration was determined by measuring the total concentration in a 20 μl sample from the dialysis tubing and subtracting the free Ca^{2+} concentration.

DTNB REACTION

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) reacts with free sulfhydryl groups of proteins to form thionitrobenzoate protein and liberating one mole of thionitrobenzoate anion for each -SH group (figure 2.4). This strongly colored anion can be detected by its high absorption at 412 nm (Means & Feeney, 1971). Titration of calponin with the thiol specific reagent DTNB, was used to determine the number of sulfhydryls exposed in the benign solvent 50 mM MOPS pH 7.15, 100 mM NaCl, and 1 mM EGTA, which had the DTT removed by running the

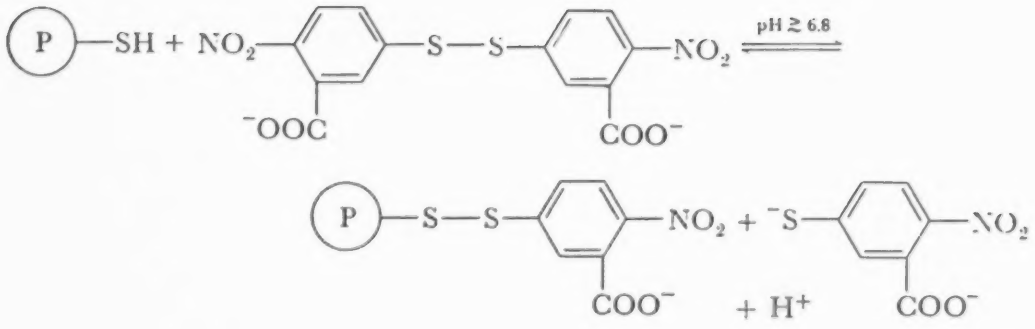


Figure 2.4: DTNB reacts with free sulfhydryl groups of proteins, forming thionitrobenzoate protein, and liberating for each -SH group 1 mol of thionitrobenzoate anion.

sample over a gel filtration column. Stock DTNB was made up at 10 mM in 50 mM $NaPO_4$, pH 7.2. The absorption spectrum of calponin was first determined to establish the protein concentration, then a 10 fold excess of DTNB was added to the blank and protein containing cell. The increase in absorption at 412 nm was monitored to determine the concentration of free sulfhydryls. The released thiobenzoate ion was quantitated using the $A^{1M}_{412nm} = 13600$ (Means and Feeney, 1971).

ATPASE ASSAYS

ATPase assays to compare the activity of native calponin with acrylodan calponin were performed using 0.125 mg/ml rabbit skeletal myosin, 0.5 mg/ml rabbit skeletal actin, 5 mM $MgCl_2$, 60 mM KCl, 25 mM Tris pH 7.5, 0.5 mM DTT. The reaction was started by adding 2 mM ATP and ended after five minutes by the addition of TCA. Inorganic phosphate was measured using the colorimetric assay with malachite green according to Itaya and Ui (1966).

ATPase assays to compare the efficacy of calmodulin, S-100b, and caltropin in regulating calponin were performed using 1 μM S1 and 7 μM actin, 3.5 μM calponin, and either 3.5, 7.0, or 21.0 μM of the relevant calcium binding protein. The buffer used was 25 mM Tris, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT in a 500 μl reaction volume. All of the glassware was acid washed so the free calcium concentration in the buffer was below the level at which calponin interacts

with calcium binding proteins for the experiments in the absence of calcium. To determine the activity in the presence of calcium, 3 mM $CaCl_2$ was added to the reaction tube. The reaction was started by adding 1 mM ATP, and ended after five minutes by the addition of TCA. Inorganic phosphate was measured as above.

COMPUTER ASSISTED ANALYSIS

The secondary structure predictions from the far UV CD spectra of calponin, calmodulin, and the calponin-calmodulin complex were determined using the FORTRAN program CONTIN, developed by Provencher and Glöckner (1981) which analyzes CD spectra as a combination of CD data collected from 16 proteins whose structures are known from x-ray crystallography.

Curve fitting of fluorescence titrations to predict Kd's was accomplished by fitting the data to a cubic equation describing the reaction $P + L \rightleftharpoons PL + L \rightleftharpoons PL_2$, in which two ligands (L) bind to one target molecule (P) (Williams *et al.*, 1986). The dissociation constants, as well as the fractional fluorescence change attributable to each ligand binding, were allowed to be determined individually for each titration. The reported range of values include the mean +/- the standard deviation.

Free Ca^{2+} concentrations were calculated by an iterative method described by Perrin and Sayce (1967) using a program written for the Apple Macintosh (courtesy of Dr. B.D.Sykes and R. Boyko, Department of Biochemistry, University

of Alberta). This program includes constants for binding of Ca^{2+} to the protein. The sum of the negative log of the individual binding constants used were as follows: EGTA + H ⇌ EGTA·H, 9.58; EGTA·H + H ⇌ EGTA·H_2, 8.97; EGTA·H_2 + H ⇌ EGTA·H_3, 2.8; EGTA·H_3+ H ⇌ EGTA·H_4, 2.12; EGTA + Ca ⇌ EGTA·Ca, 10.96; EGTA·H + Ca ⇌ EGTA·H·Ca, 5.33 (Golosinska *et al.*, 1991); Calm + 2Ca ⇌ Calm·Ca_2, 13.4; Calm·Ca_2 + 2Ca ⇌ Calm·Ca_4, 11.4 (Wang, 1985). Ca^{2+} titration data was fitted to a sigmoidal curve equation using Tablecurve 3.1 (Jandel Scientific).

The accessibility plot of calponin was calculated using the values compiled by Janin (1979), according to the method of Parker *et al.* (1986) wherein the average surface hydrophilicity is the mean of the profile values, and any residue having a profile value greater than 25% of the difference between the mean and the maximum value was determined to be exposed. The surface profile values were determined by summing up the accessibility value for each residue of a 7 residue segment and assigning that value to the fourth residue in the segment. These values were plotted against the amino acid residue number.

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Chapter 3

CALPONIN PURIFICATION, CHARACTERIZATION, AND INTERACTION WITH ACRYLODAN LABELLED CALCIUM BINDING PROTEINS

INTRODUCTION

Several proteins have been isolated from smooth muscle in an attempt to elucidate the complete mechanism of smooth muscle regulation. In particular, the isolation of calponin from avian smooth muscle as a heat stable protein was first described by Takahashi *et al.* in 1986. The isolation procedure involved boiling chicken gizzards prior to extraction in a high ionic strength buffer (figure 3.1). The crude protein was dialyzed into a urea buffer, and remained in urea solvent through cation exchange, concentration, and gel filtration steps in order to obtain the purified calponin. The concentration step, effected in a model YM-10 Amicon concentrator in 6 M urea @ 4°C in order to reduce the volume in preparation for the gel filtration column, proved to be too time consuming. Therefore, a modification chosen in our laboratory involved dialysis and lyophilization of the pool from the SP-Sephadex C50 column, followed by reversed phase HPLC as the second and final purification step. The calponin produced in this manner was not pure, and the gel filtration step was reinstated. In the process, however, it was discovered that calponin eluted as two closely spaced peaks off the reversed phase column at ~40% acetonitrile (figure 3.2). Since at that time it had been reported that calponin run on two dimensional gels (Takahashi *et al.*, 1986, 1988) showed a number of charged species, and was believed to be expressed as a

All steps performed @ 4°C.
Trim gizzards of connective tissue, boil in H_2O bath for 2 min., chill on ice.

↓

Homogenize in 3.5 vols. extraction buffer: 300 mM KCl, 1 mM DTT, 5 mM EGTA, 50 mM Imidazole-HCl pH 6.9, 0.5 mM phenylmethyl sulfonyl fluoride, 10 μg/ml leupeptin, 1mM sodium tetrathionate.
Centrifuge 150 000 x g for 30 min.

↓

Add 16.4 g/100 ml ammonium sulfate (30%) to the supernatent.
Centrifuge 12 000 x g for 30 min.

↓

Dissolve pellet in buffer A: 6 M urea, 50 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, 20 mM Tris-HCl pH 7.8.

↓

Dialyze twice against 1 l of buffer A for a total of 8 hrs.
Clarify by centrifuging 150 000 x g for 1 hr.

↓

Apply to a 2 x 25 cm SP-Sephadex C50 ion exchange column in buffer A.

↓

Elute with 2 x 250 ml gradient 50 - 300 mM KCl. Elutes at ~ 80-100 mM KCl.

↓

Concentrate in an Amicon YM-10

↓

Apply to a 2.6 x 90 cm Ultrogel ACA 44 column in buffer B: 6 M urea, 400 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, 0.02 % NaN_3, 20 mM Tris-HCl pH 7.5.

↓

Dialyze calponin fraction against 2 x 4 l of 75 mM Tris-acetate pH 9.3.
Clarify by centrifuging at 150 000 x g for 1 hr.

↓

Dialyze supernatent into 20 mM Tris-HCl pH 7.0, 0.1 mM EGTA, 0.5 mM DTT.
Store at 0°C for use within 48 hrs.

Figure 3.1: Purification of Calponin according to Takahashi *et al.* (1986, 1988).

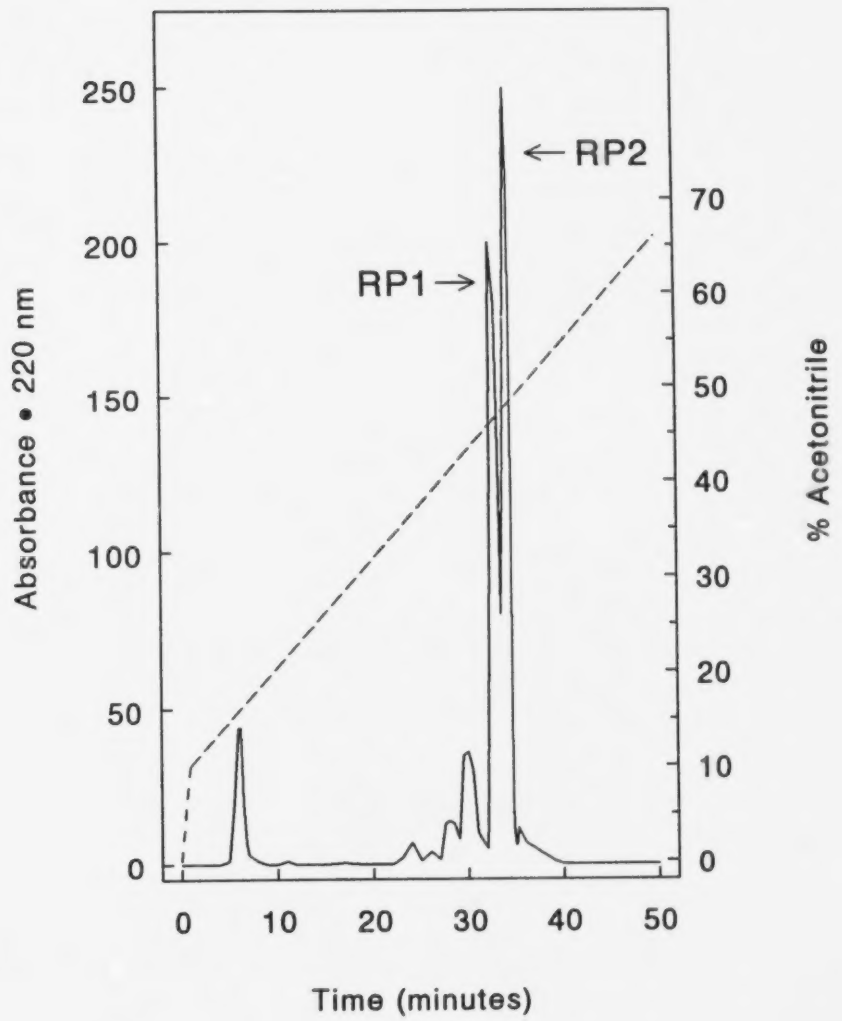

Figure 3.2: Profile of reversed-phase chromatography of calponin. Calponin from the SP-Sephadex column was loaded onto a Synchropak C18 column. A 1%/min, 10-70% acetonitrile gradient (---) was used and 3 ml/min. The wavelength of detection was 220 nm.

number of isoforms with different pI's, the characterization of these two peaks was undertaken to see if they represented different isoforms of calponin.

RESULTS OF EXPERIMENTS WITH CALPONIN ISOFORMS

Amino Acid Composition of Calponin.

The amino acid compositions of RP1 and RP2 are presented in table 3.1. The compositions of the two isoforms are very similar, with significant differences being apparent for only valine and arginine. They compare closely with the composition published for whole calponin by Takahashi *et al.* (1988). These results do not provide strong evidence for RP1 and RP2 being isoforms of highly variant composition.

Far ultraviolet circular dichroism spectra of RP1 & RP2.

Circular dichroism spectra in the far UV (250-200 nm) region are characteristic of the secondary structure present in proteins. The spectrum of both proteins is characterized by a minimum at 207 nm, and a broad shoulder around 221 nm, although the absolute ellipticities differ (figure 3.3). RP1 has slightly less negative ellipticity with $[\theta]_{221nm}$ = -7840+/-300°, while RP2 demonstrated a $[\theta]_{221}$ of -8310+/-300°. While the absolute ellipticites may vary +/- 300° due to experimental error, it should be noted that for four different preparations, RP2 consistently had a slightly greater negative ellipticity than RP1.

Table 3.1: Amino Acid composition of calponin expressed as % of total residues

Amino Acid	RP1	RP2	calponin[a]	calponin[b]
Aspartic acid/Asparagine	11.3	11.4	11.3	12.2
Threonine	5.3*	5.3*	5.6	5.3
Serine	4.2*	4.9*	4.7	4.4
Glutamic acid/ Glutamine	13.2	12.9	15.1	13.4
Proline	4.9	4.9	3.9	4.7
Glycine	10.9	11.0	11.3	10.8
Alanine	6.8	6.8	6.5	7.0
Cysteine	1.4+	1.4+	N.D.	N.D.
Valine	**6.1**	**3.8**	4.1	4.3
Methionine	3.0*	3.0*	2.6	2.6
Isoleucine	4.2	4.2	4.5	4.3
Leucine	8.7	8.4	8.5	8.5
Tyrosine	3.4	3.4	3.8	3.5
Phenylalanine	3.4	3.4	3.0	3.2
Histidine	2.3	2.3	2.1	2.3
Lysine	6.8	6.8	7.4	7.0
Arginine	**5.7**	**4.9**	5.0	5.3
Tryptophan	0.7*	0.7*	0.7*	N.D.

[a]Calponin: from Takahashi *et al.* (1988)
[b]Calponin: purified in this lab
*Determined from hydrolysis in methanesulphonic acid at 160°C for 45 min.
+Determined by adding 2% DMSO to 6M HCl and hydrolyzing at 110°C for 24 hrs.
N.D. = not determined

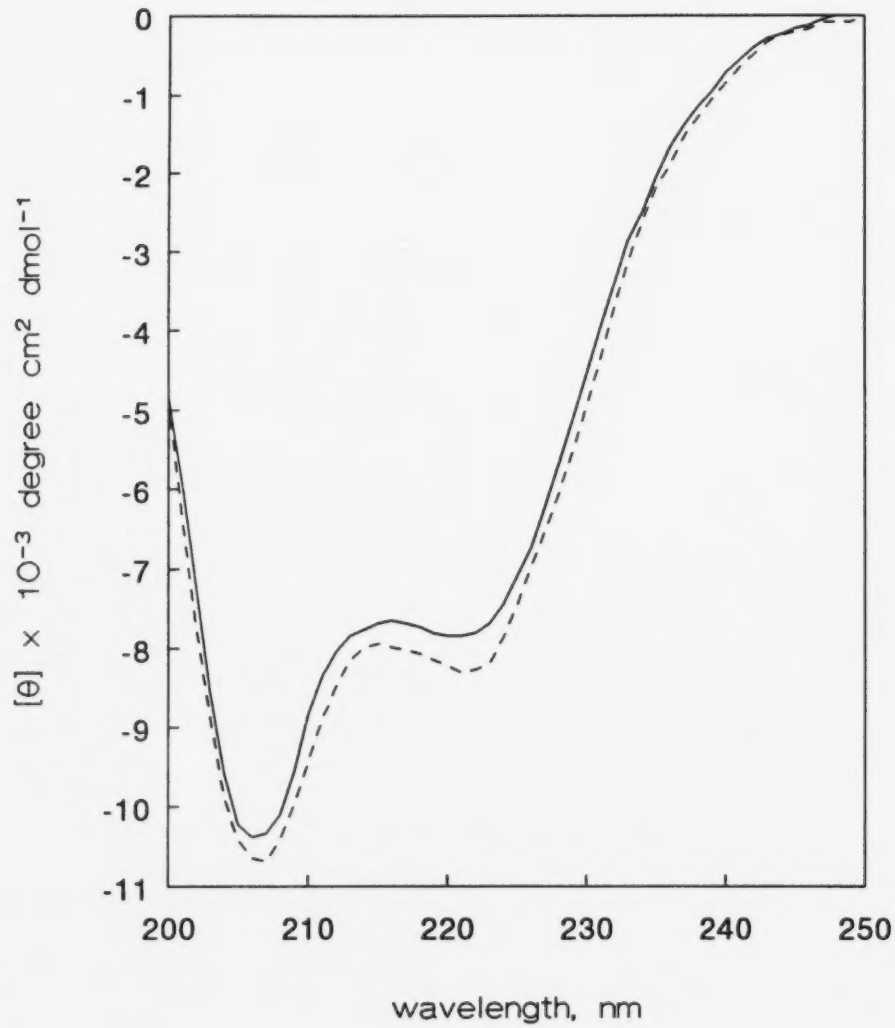

Figure 3.3: Far ultraviolet CD spectra of RP1 (——) and RP2 (---). Protein concentrations were 0.92 mg/ml for RP1 and 1.25 mg/ml for RP2 in 25 mM Tris-HCl pH 7.5, 100 mM NaCl, and 1 mM DTT. The path length was 0.05 cm.

Near ultraviolet circular dichroism spectra of RP1 & RP2.

The near UV circular dichroism spectra (350-250 nm) are representative of the aromatic amino acids phenylalanine, tryptophan, and tyrosine. The free amino acids do not display strong circular dichroic bands, however when the aromatic residues are in asymmetric environments, or in contact with other aromatic residues, characteristic spectra are produced: phenylalanine from 255 to 270 nm; tyrosine between 275 and 285 nm; and tryptophan from 280 to 300 nm. Cystine may also make a weak contribution at wavelengths above 240 nm (Strickland, 1974). Comparison of the near UV spectra of the two isoforms (figure 3.4) shows slight differences even though the amino acid composition indicates RP1 and RP2 have the same tryptophan, tyrosine, and phenylalanine content. The differences in the spectra would therefore suggest minor differences in the environment of these chromophores between the two isoforms. Once again the differences, while slight, were consistent over four different preparations.

Stokes Radius of Calponin.

Gel filtration revealed differences in the Stokes radius between the two isoforms (figure 3.5). RP1 has a radius of 20.9 Å, while RP2 has a radius of 23.1 Å. Calponin, which had not been fractionated into these two isoforms, eluted as a broader peak centered at 22.1 Å under our conditions, presumably representing an average of the two isoforms. This difference in Stokes radius does not

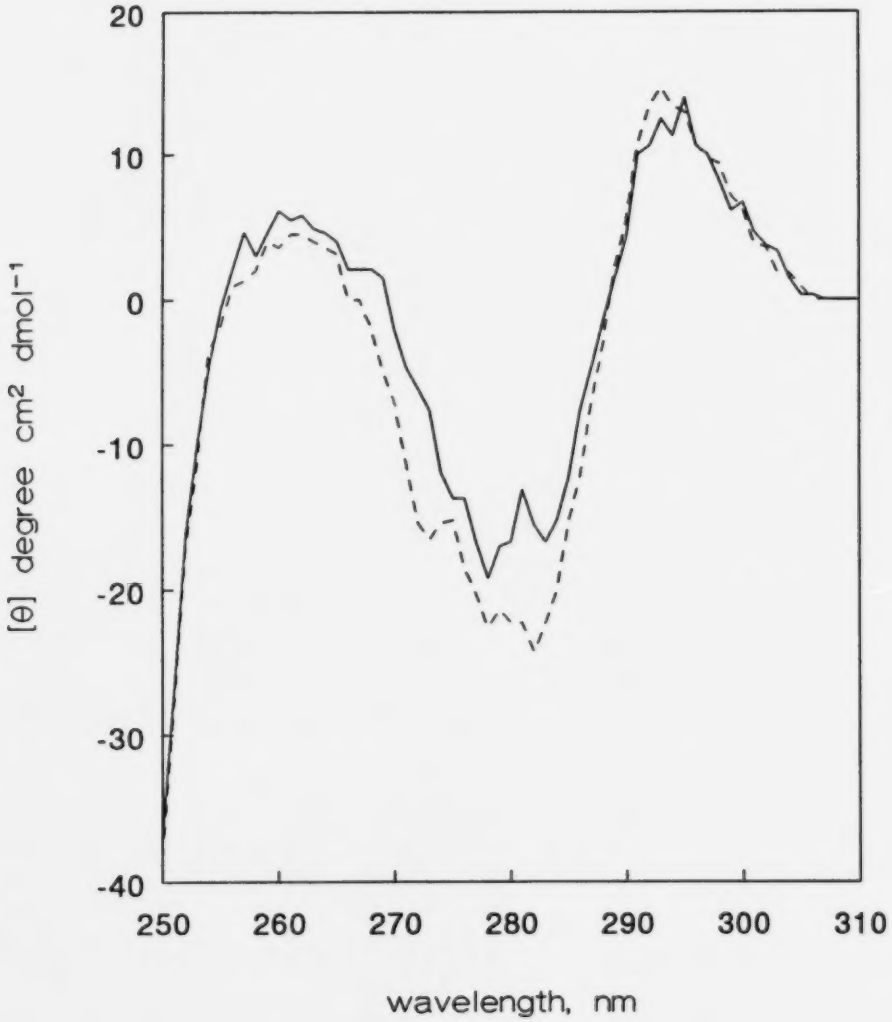

Figure 3.4: Near ultraviolet CD spectra of RP1 (——) and RP2 (---). Protein concentrations were 0.55-0.56 mg/ml in 25 mM Tris-HCl pH 7.5, 100 mM NaCl, and 1 mM DTT. The path length was 1 cm.

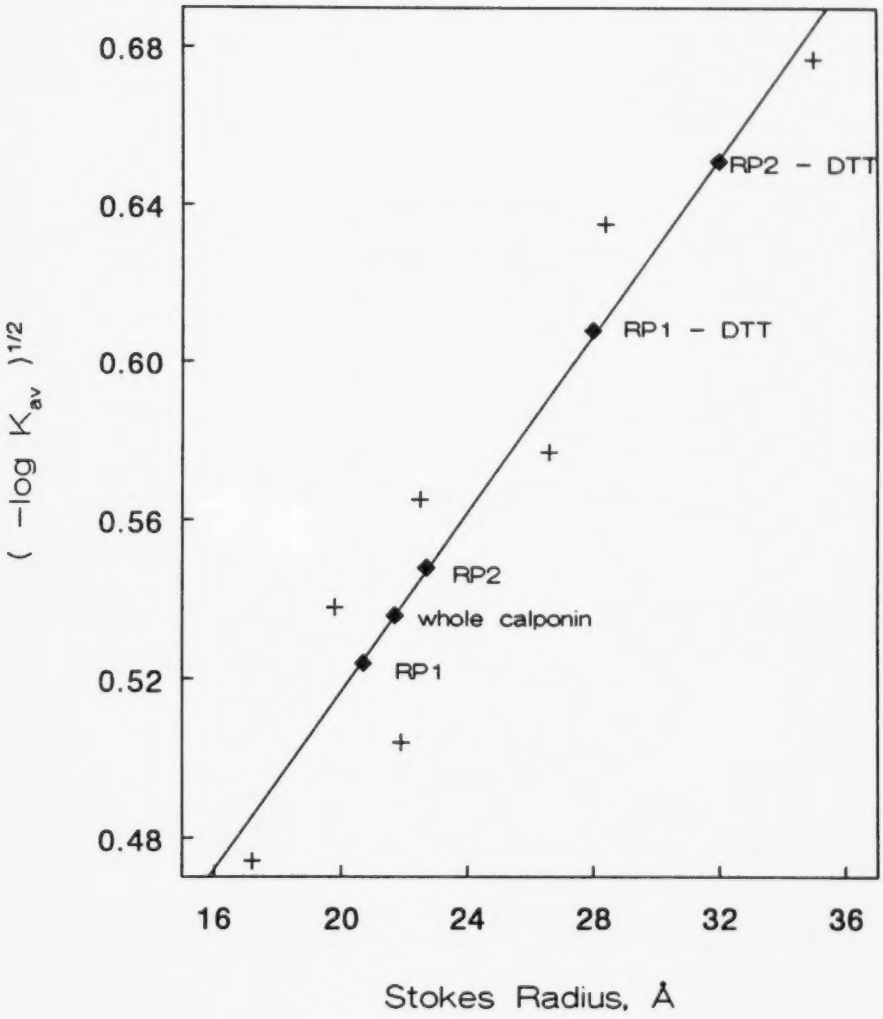

Figure 3.5: Determination of Stokes radius for RP1, RP2, native calponin, and oxidized samples of calponin. The buffer conditions were 50 mM MOPS pH 7.2, 100 mM NaCl, and 1 mM DTT, except for the oxidized samples where the DTT was omitted.

necessarily mean a difference in molecular weight as RP1, RP2, and whole calponin cannot be differentiated by SDS-PAGE (figure 3.6). However, it does suggest a slight difference in molecular size. The theoretical Stokes radius for an equilvalent sphere the molecular weight of calponin is 21.05 Å, indicating calponin is a globular protein. Takahashi *et al.* (1986) found a Stokes radius for calponin of 27.1 Å using an identical column and similar eluting conditions. There is no obvious rationale for this discrepancy. The calponin recovered from the gel filtration column was identical by SDS-PAGE to that loaded onto the column (data not shown). It should be noted, however, that when the buffer used for the gel filtration column did not contain DTT, Stokes radii of 28 Å and 32 Å for RP1 and RP2 respectively, were found, apparently representing more oxidized forms of the protein (figure 3.5). A greater percentage of RP1 over RP2 became oxidized as observed by the increase in Stokes' radius indicating RP1 was more subject to oxidation. This suggests a difference in the cysteine environment between the two isoforms.

SUMMARY OF RESULTS ON ISOFORMS

The differences between these two isoforms of calponin are limited to slight variations in secondary structure and molecular size. Since the isoforms are separated on a reversed phase column, some conformational variation resulting in hydrophobicity differences probably exists.

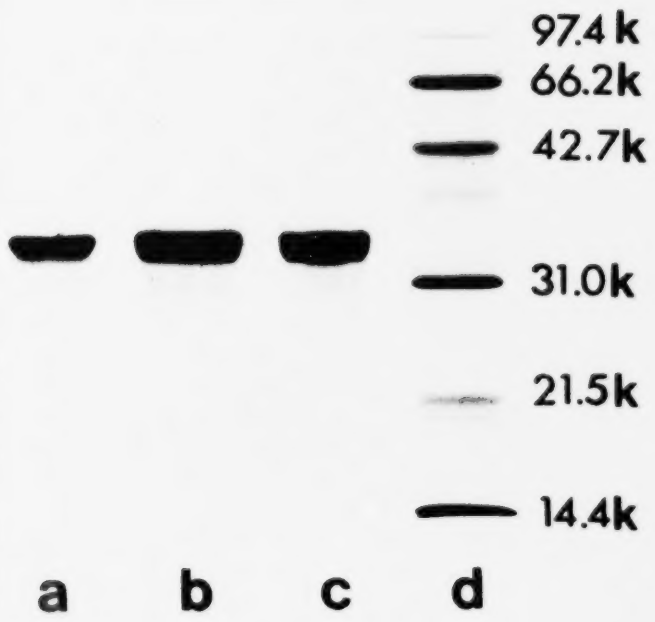

Figure 3.6: SDS-PAGE of Calponin. (a) Calponin purified on hydroxylapatite; (b) RP1 isoform; (c) RP2 isoform; (d) molecular weight standards.

This method not only produced small amounts of the protein, but also the calponin was unstable and subject to extensive proteolysis as seen by SDS PAGE and experiments in the analytical ultracentrifuge. As well, the resulting protein was not soluble at concentrations above 1 mg/ml, and was subject to precipitation upon gentle agitation as seen by the formation of a white cloud in the cuvette during spectroscopic experiments. For all of these reasons, an attempt to streamline the preparation and isolate a more stable calponin was undertaken.

ALTERATIONS TO PREPARATION METHODOLOGY

The flow sheet in figure 3.7 outlines the modified preparative methodology adopted. In the course of designing protocols to improve the calponin preparation methodology, two areas were addressed: speeding up the preparation, and reducing the amount of time calponin spends in urea solvent. To this end, the SP -Sephadex C50 column was replaced with 2.5 x 30 cm S-Sepharose fast flow column equilibrated in urea solvent from which calponin eluted at ~200mM NaCl (figure 3.8). This column was run at a flow rate of 200 mls/hr, and calponin can be loaded and eluted within one day. The pH at which this column was run was dropped from pH 6.0, which Takahashi *et al.* (1986) used, to pH 4.5 in acetate buffer in order to improve binding to the column; however, this also slowed modification of lysine residues by carbamylation due to the degradation of urea into ammonium cyanate (Means & Feeney, 1971). Following this cation exchange

All steps performed at 21°C unless otherwise noted.
Trim gizzards of connective tissue, homogenize in 3 vols. low ionic strength buffer: 40 mM Tris-HCl pH 7.5, 80 mM NaCl, 2 mM EDTA, 0.05% NaN_3, 1 mM PMSF, 1 mg aprotinin, 5 mg leupeptin, 5 mg pepstatin.

↓

. Spin 16 500 x g for 45 minutes → Supernatent used for caltropin preparation.
Suspend pellet in high ionic strength extraction buffer of Takahashi *et al.* (1986)
Bring suspension to 90°C, then cool on ice.
Centrifuge @ 4°C, 17 700 x g, for 45 min.

↓

Add 16.4 g/100 ml ammonium sulfate (30%) to the supernatent.
Centrifuge @ 4°C, 17 700 x g, for 30 min.

↓

Dissolve pellet in buffer A: 6 M urea, 50 mM NaCl, 2 mM EGTA, 0.5 mM DTT, 25 mM Na Acetate pH 4.5, 0.01% NaN_3.

↓

Dialyze against 2 x 14 l of buffer A for a total of 16 hrs @ 4°C.
Clarify by centrifuging 165 000 x g for 45 min.

↓

Apply to 2.5 x 20 cm S-Sepharose Fast Flow ion exchange column in buffer A.

↓

Elute with 2 x 400 ml gradient 50 - 400 mM NaCl. Elutes at ~ 200 mM NaCl.

↓

Dialyze 2 x 14 L against buffer B: 5 mM $NaPO_4$ ph 6.0, 100 mM NaCl, 1 mM DTT, 0.3 mM $CaCl_2$ for a total of 16 hrs @ 4°C.

↓

Apply to a 2.5 x 30 cm Biorad Hydroxylapatite column in buffer B.
Elutes in the void volume.

↓

Dialyze calponin fraction against 2 x 14 l H_2O pH 5.0 @ 4°C, and lyophilize

↓

Suspend in 0.1% TFA, and apply to 1.0 x 25 cm C18 RP column.
Elutes at ~ 40 % acetonitrile. Lyophilize.

Figure 3.7: Purification of Calponin according to modifications adopted.

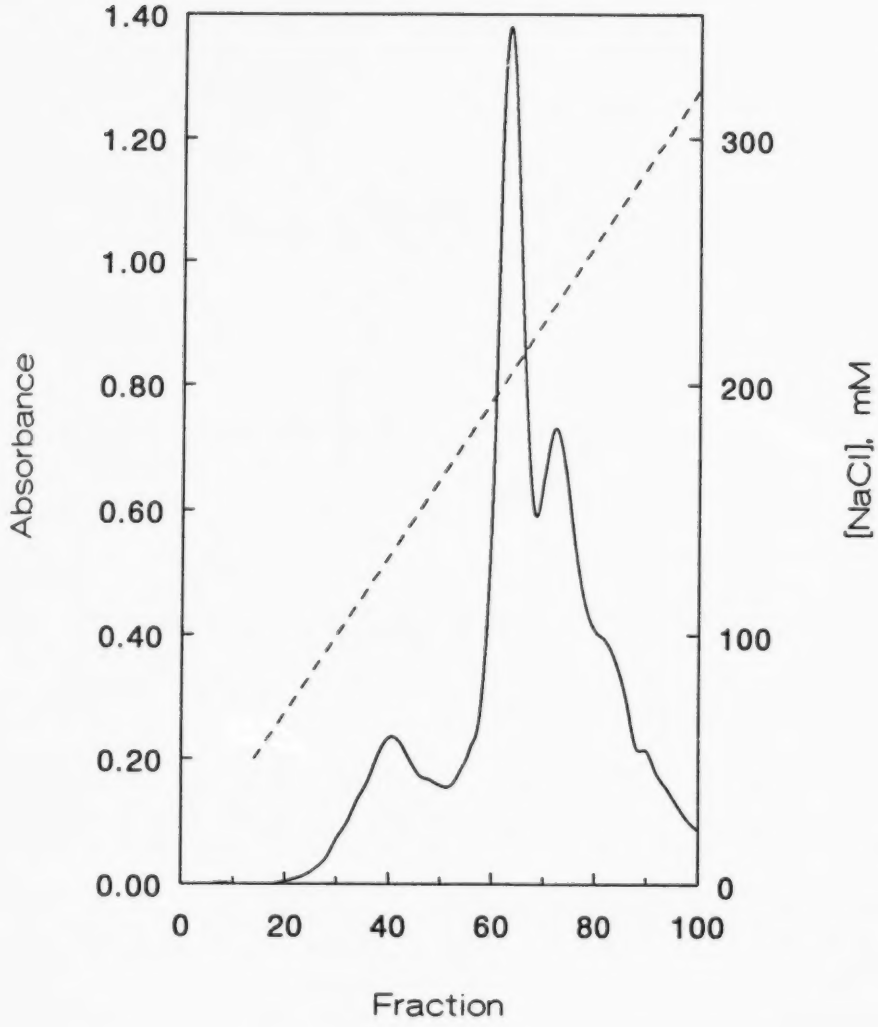

Figure 3.8: S-Sepharose Fast Flow profile of calponin purification. Crude protein extract was applied in 6 M urea, 25 mM NaAc pH 4.5, 50 mM NaCl, 0.5 mM DTT, 2 mM EGTA, and 0.01% NaN_3. The 87.5 mM/hr NaCl gradient is shown (---).

step, the protein was dialyzed before being applied to a hydroxylapatite column in a non urea buffer, from which it eluted in the void volume. This step removes tropomyosin contamination. Finally, calponin was applied to a C18 reversed phase column, from which it eluted in a single peak at ~ 40 % acetonitrile. Not only does the protein no longer separate into two distinct peaks, but it is more stable, with concentrations of 2-3 mg/ml being obtainable in solution. It also exhibited much greater resistance to proteolysis, and virtually no precipitation occurred during spectroscopic experiments. This information, taken together with comments that even cloned calponin can produce multiple spots on two dimensional gels, indicates that a variety of charged species are present and may be due to post-translational modifications (J.P. Jin, personal communication). This suggests the origin of the multiple isoforms we observed was probably modification of the protein either through: carbamylation due to the long exposure to urea at neutral pH with low concentrations of Tris-HCl present to act as a scavenger, or oxidation of the cysteines due to insufficient sulfhydryl protection, or a combination of these two events. The oxidation possibility is exemplified by the gel filtration data where the Stokes radius increases if the protein is insufficiently reduced. The physical experiments performed more recently on this more stable calponin do not differ in any significant manner from the results obtained working with the two isoforms. Therefore, while the protein is now more stable and easier to work with, it does not appear that its structure was severely affected by these modifications. Based upon the similarity of the RP2 far UV CD spectrum to that of the calponin presently

routinely isolated, together with the fact that RP2 was the more stable of the two isoforms, and was obtained in greater quantities, RP2 seems the likely candidate for the native calponin, while RP1 likely had been modified during the preparative procedure. Mass spectroscopy of calponin purified by this modified procedure confirmed the molecular mass to be the same as that originally described by Takahashi *et al.* (1986).

pH STABILITY OF CALPONIN

In order to determine optimum conditions for analytical work with calponin a pH denaturation profile was determined by observing the effect upon the ellipticity of calponin at 221 nm of changing the pH over the range 1.5 to 11.5. It is evident from figure 3.9 that the calponin structure is stable over a range of pH from 6 to 10; therefore we chose to perform our experiments under the approximately physiological conditions of pH 7.0-7.2, and 100 mM NaCl.

ACRYLODAN LABELLED CALCIUM BINDING PROTEINS

It has been established that calponin is able to interact with both calmodulin and troponin C in a calcium dependent manner (Takahashi *et al.*, 1986, Takahashi *et al.*, 1988). Abe *et al.* (1991), and Makuch *et al.*(1992) also showed that the interaction of calponin with calmodulin was able to release calponin's inhibition of

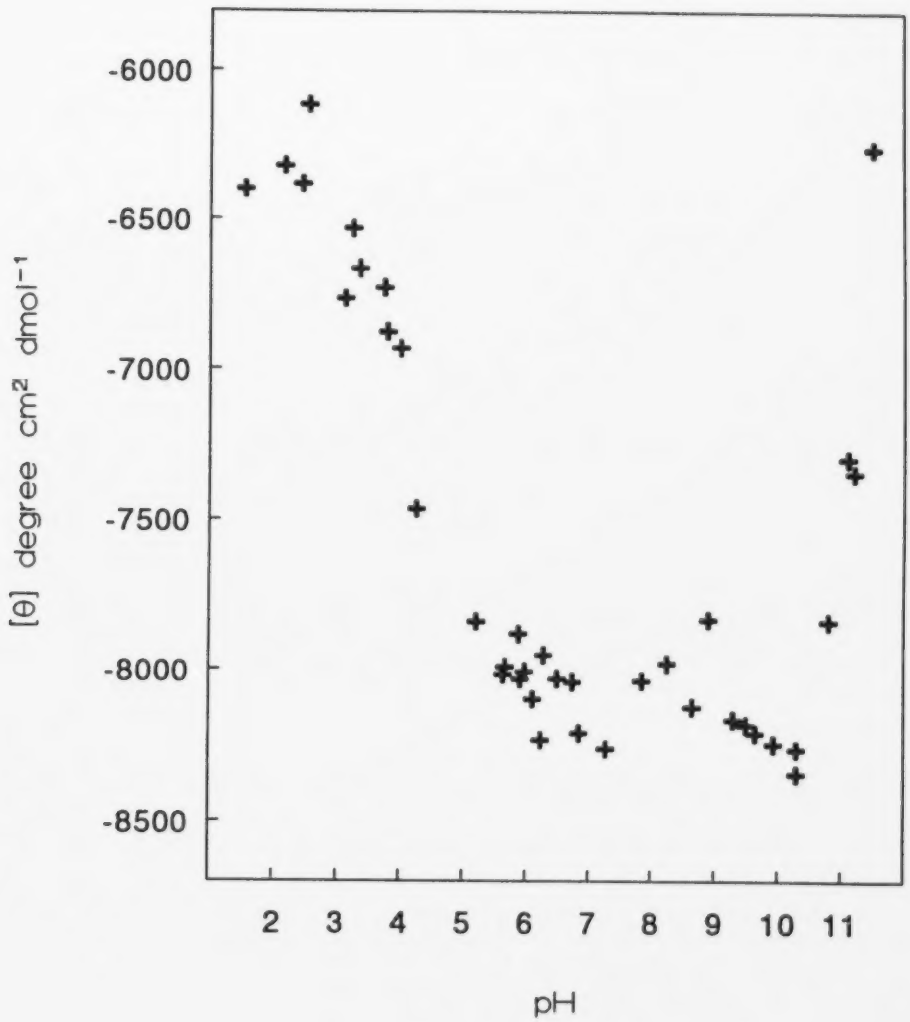

Figure 3.9: pH Titration of the circular dichroic ellipticity of calponin monitored at 221 nm. The buffer was 10 mM $NaPO_4$, 100 mM NaCl, and 1 mM DTT.

the actin activated myosin ATPase. It was of interest therefore to characterize the interactions between calponin and these calcium binding proteins. Circular dichroism was used as a spectral method capable of detecting secondary structure changes in order to demonstrate evidence of complex formation. In addition, it has previously been demonstrated that cysteine 27 of wheat germ calmodulin (Strasburg *et al.* 1988), and cysteine 98 of rabbit skeletal troponin C (Wang *et al.*, 1987) can be labelled with fluorescent probes to monitor the behavior of these proteins. S-100b also has cysteines at positions 68 and 84. Although S-100b has two cysteines,it is possible to selectively label this protein at a single cysteine with N-(1-pyrenyl) iodoacetamide, by carrying out the reaction in the presence of EDTA (Mani & Kay, 1985), and acrylodan would presumably label in the same fashion. Therefore, labelling these proteins with the fluorescent probe acrylodan was undertaken. The properties of acrylodan have been described previously by Prendergast *et al.* (1983) and include specificity for cysteine residues, and a very high sensitivity to the polarity of the environment of the probe with resultant large wavelength shifts. Using these tools, we were indeed able to demonstrate fluorescent effects of these proteins upon interaction with calponin.

RESULTS OF EXPERIMENTS WITH ACRYLODAN CALMODULIN

Circular dichroism of calponin complexed with calcium binding proteins.

Circular dichroism spectroscopy is used to monitor secondary structure

changes in proteins. As such it can be a useful monitor of complex formation if changes in structure occur upon interaction. In order to determine if labelling the calmodulin with acrylodan altered the structure of calmodulin, the spectrum of the labelled protein was compared to the spectrum of native calmodulin, to ensure no significant changes had occurred (data not shown). The circular dichroism spectra of calponin in complex with calmodulin, troponin C, and with S-100b were determined individually and compared to the theoretical spectra of these proteins in combination, assuming no secondary structural changes occurred. Figure 3.10 shows the circular dichroic spectrum of the calponin-calmodulin complex in the presence of calcium, compared to the theoretical spectrum obtained by summing the contributions of the individual constituents. From this figure it can be seen that the complex has 440° more negative ellipticity at 221 nm than the theoretical complex when calcium is present. This compares with only a 113° ellipticity difference at 221 nm in the absence of calcium which falls within the experimental error of this technique (+/- 300°). Table 3.2 compiles the ellipticity values of the complexes of calponin with the calcium binding proteins, and the calculated ellipticity values in the presence of calcium for a theoretical complex. It can be seen that in all three cases differences in the observed spectra compared to calculated are seen, indicative of a small change in secondary structure and complex formation.

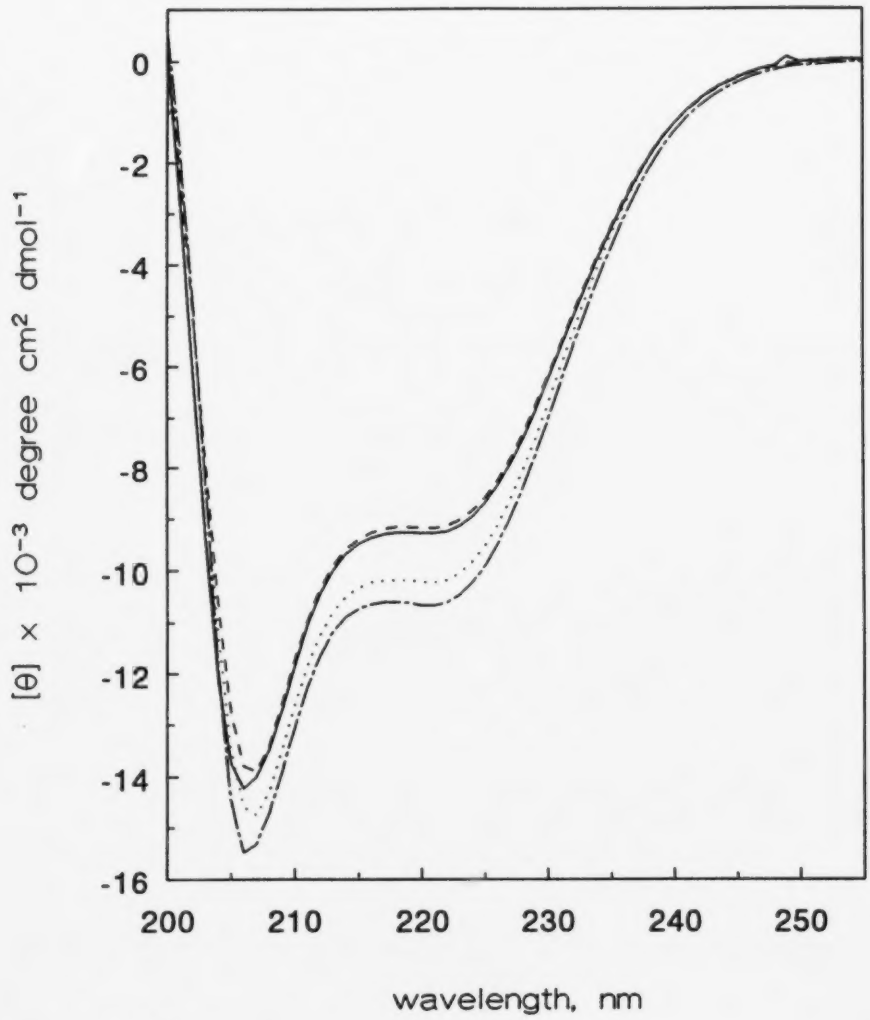

Figure 3.10: Circular dichroism spectra of the observed 1:1 complex of calponin with acrylodan calmodulin in the absence of calcium (——), in the presence of calcium (—— —), and the calculated complex in the absence (---) and presence (• • • •) of calcium. The buffer used was 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, +/- 3 mM $CaCl_2$.

Table 3.2: Mean residue ellipticities of the complexes of calponin with the CaBP's.[a]

Complex containing:	[Θ] observed	[Θ] theoretical	[Θ] difference[b]
Acrylodan CM	-10670	-10230	-440
Acrylodan TNC	-11300	-10360	-940
Acrylodan S-100b	-11840	-12290	+450

[a] All ellipticity values quoted are at 221 nm.
[b] The difference is [Θ] observed - [Θ] calculated

Fluorescence Studies with the acrylodan labelled calcium binding proteins.

Acrylodan calmodulin, excited at 366 nm, has a peak wavelength of emission of 520 nm, as seen in figure 3.11. This acrylodan fluorescence is sensitive to interaction both with calcium, and with calponin in the presence of calcium. Calcium causes a decrease in fluorescence and a change in peak wavelength to 522 nm, while the subsequent addition of calponin increases the fluorescence at 460 nm, the wavelength of greatest change, 15-fold, and shifts the peak wavelength to 490 nm, indicating the acrylodan probe has moved into a less polar, more buried environment. The acrylodan fluorescence of troponin C and S-100b also respond to interaction with calcium and with calponin in a calcium sensitive manner, and the details of these fluorescence spectra are presented in table 3.3.

Calcium titration of the calmodulin-calponin complex.

These changes in the fluorescence of the acrylodan labelled calcium binding protein upon interaction with calponin can be exploited in order to monitor the calcium dependence of complex formation. Figure 3.12 shows the relative fluorescence change as a function of pCa^{2+} for native calmodulin, acrylodan labelled calmodulin, and the calponin-acrylodan-calmodulin complex. Since the fluorescence increases upon complex formation, the curve represents the calcium dependence of complex formation. Native calmodulin has a pCa midpoint of 6.2,

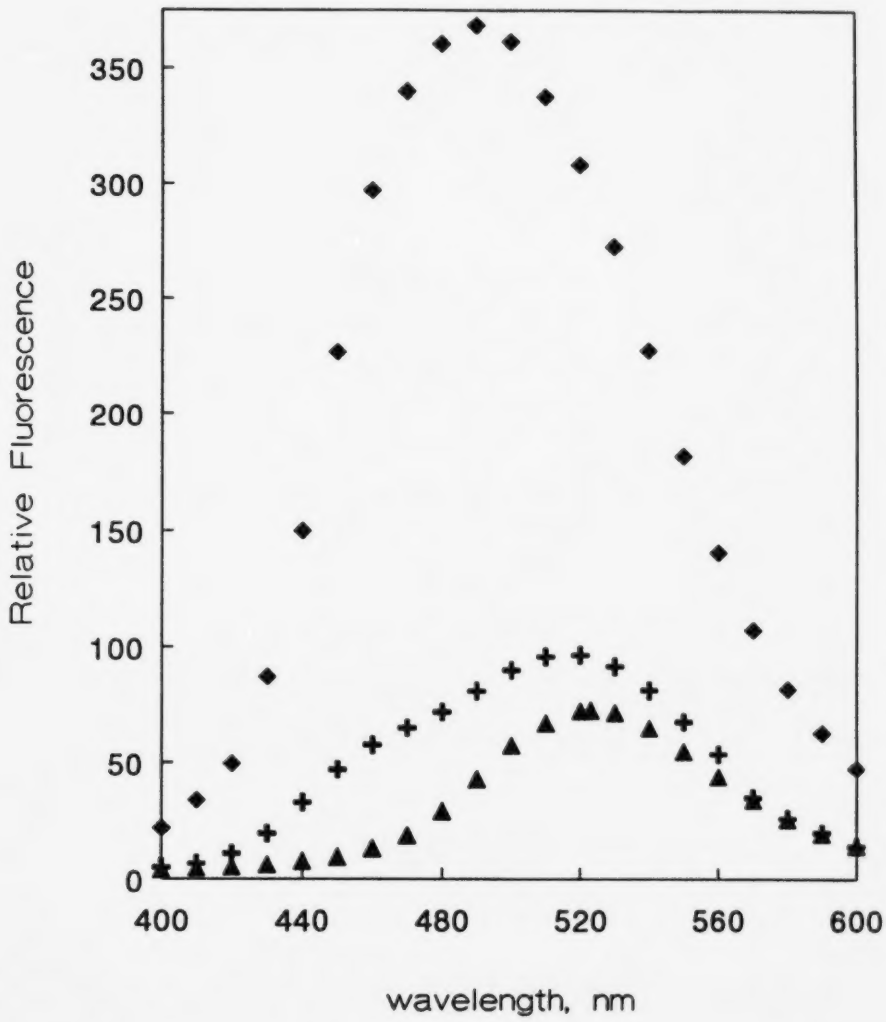

Figure 3.11: Fluorescence emission spectra of acrylodan calmodulin (+), acrylodan calmodulin in the presence of calcium (▲), and in the presence of calcium and calponin (♦). The buffer conditions were 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, ±3 mM $CaCl_2$. The excitation wavelength was 366 nm.

Table 3.3: Fluorescence Properties of acrylodan labelled calcium binding proteins[a].

Protein	Apo State	+ Ca^{2+}	+ Calponin	Fl change
Acrylodan CM	520	522	490	↑15x
Acrylodan TNC	505	505	482	↑4.4x
Acrylodan S-100b	495	490	472	↑2.2x

[a]The wavelength of peak fluorescence emission for each protein is shown. The excitation wavelengths are as described in the text. The fluorescence change is the increase in fluorescence emission when monitored at 460 nm, since in all cases this was the wavelength of greatest change.

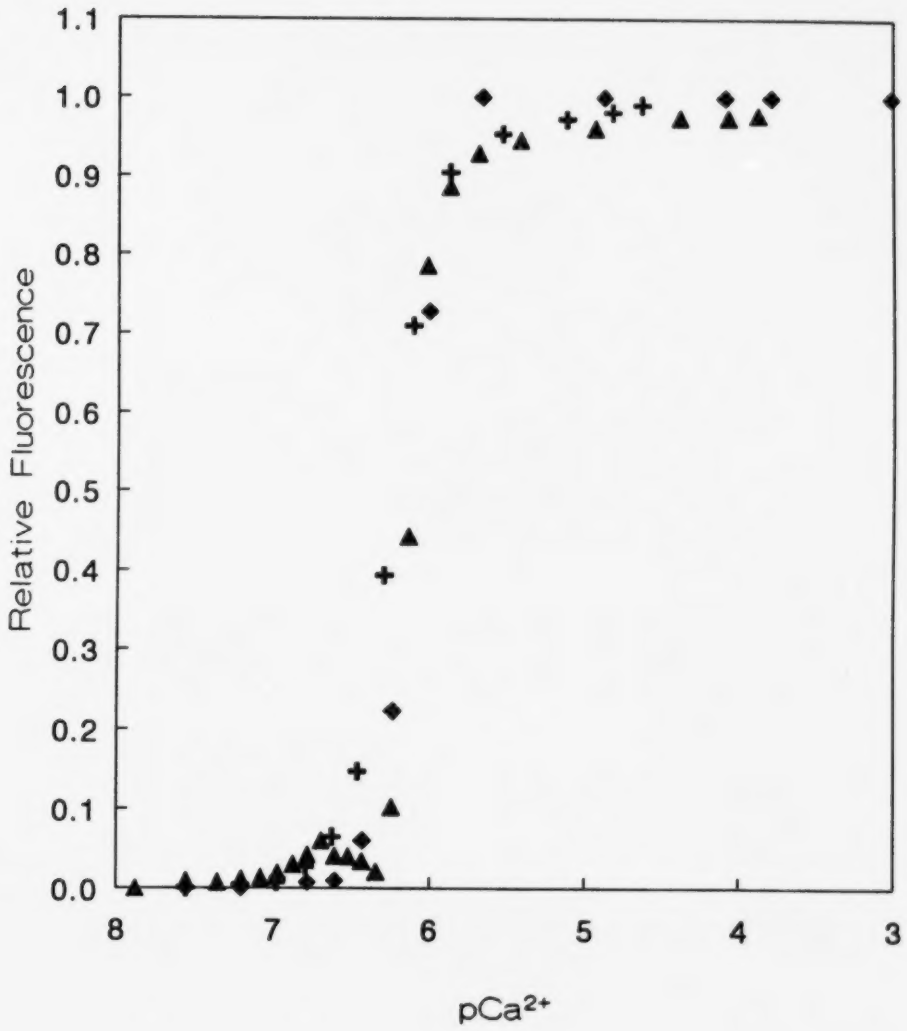

Figure 3.12: Fluorescence titration of native calmodulin (+), acrylodan calmodulin (▲), and the acrylodan calmodulin - calponin complex (♦) with calcium. Excitation was at 277 nm with emission monitored at 305 nm for native calmodulin and at 366 nm, with emission monitored at 460 nm for acrylodan calmodulin and the complex. The buffer was comprised of 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA.

which presumably represents contributions from the low and high affinity calcium binding sites. When acrylodan calmodulin is titrated with calcium, it has a midpoint at a pCa of 6.1, as does the complex of acrylodan calmodulin and calponin. This finding indicates that this method is not able to detect any change in calmodulin calcium affinity upon interaction with calponin.

Calponin titration of acrylodan calmodulin.

The increase in fluorescence upon complexation can also be used to monitor complex formation with increasing amounts of calponin added to acrylodan-calmodulin in the presence of calcium. When such a titration is performed, the increase in fluorescence does not generate a hyperbola, but rather is sigmoidal in shape with increasing calponin (figure 3.13). While this was most pronounced upon interaction with calmodulin, the trend was also apparent using S-100b or troponin C. Thus the complex formed is greater than 1 : 1, and further studies are required in order to define the stoichiometry of interaction before these results can be interpreted in terms of binding affinities.

Crosslinking of calponin and calmodulin.

In order to determine the nature of the complex, cross linking studies were attempted employing the zero length cross linker EDC and varying ratios of calponin to calmodulin in the presence and absence of calcium. Experiments were performed with equivalent amounts of calponin and calmodulin, as well as with

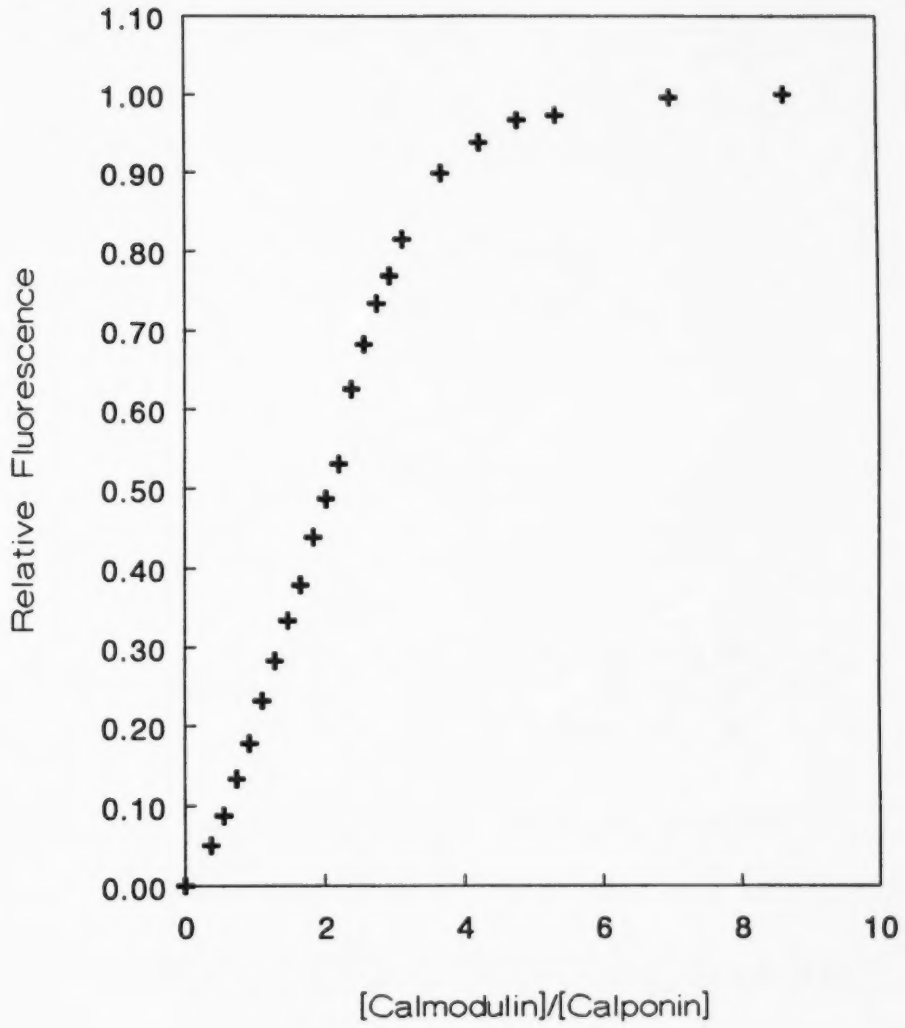

Figure 3.13: Fluorescence titration of acrylodan calmodulin with calponin. The buffer was 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 3 mM $CaCl_2$. Excitation was at 366 nm, while emission was monitored at 460 nm.

excess of both calponin or calmodulin. In particular, species of higher molecular mass than a 1 : 1 complex were evident on SDS-PAGE when excess calmodulin over calponin was combined in the presence of calcium (figure 3.14). Present are crosslinked adducts of apparent molecular weight 17 kDa, 34 kDa, 66 kDa, and 90 kDa. Due to the anomolous migration of the complexes, together with the fact that the mass of calmodulin is approximately one half the mass of calponin, the composition of these bands was difficult to define. As an alternative method these studies were carried out in the analytical ultracentrifuge, and it was found that the stoichiometry was two mols calmodulin interacting with each mol of calponin (Wills *et al*., 1993). The details and results of these experiments are amplified in chapter four.

SUMMARY OF ACRYLODAN CALMODULIN-CALPONIN INTERACTION

This ratio of interaction of two mols calmodulin per mol of calponin indicated that monitoring the labelled calmodulin was an unsuitable method for performing the titration, since initially, in the presence of excess calmodulin, small amounts of calponin would presumably each bind to two mols of calmodulin; however, as the titration progressed, and increasing amounts of calponin were added, the interaction would be reduced to 1 : 1. For these reasons, studies were initiated to label the calponin rather than the calcium binding protein, and to follow the interaction from the fluorescence changes in calponin.

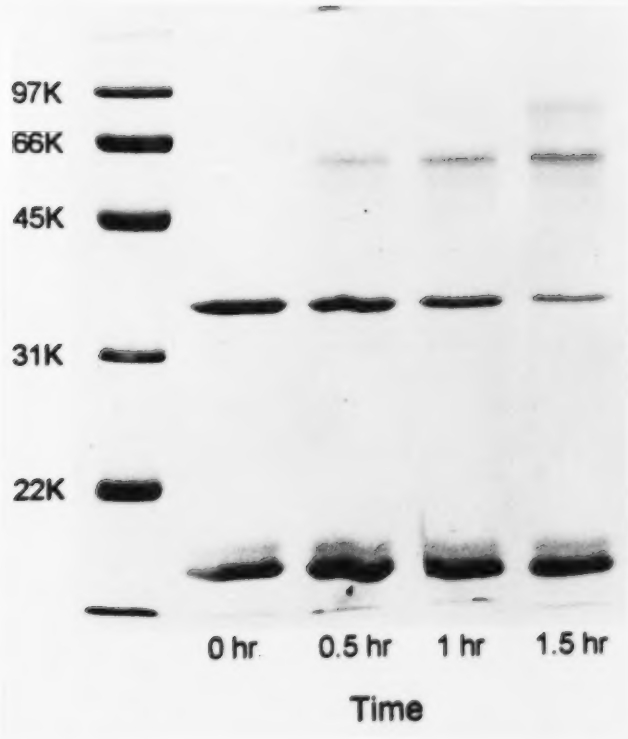

Figure 3.14: SDS-PAGE of EDC crosslinking products of calmodulin-calponin complex with increasing time. The proteins were combined in the ratio of 4.2 mols calmodulin to 1 mol calponin in 50 mM MOPS pH 7.2, 100 mM NaCl, and 1.5 mM $CaCl_2$.

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Chapter 4

CHARACTERIZATION OF THE SMOOTH MUSCLE CALPONIN AND CALMODULIN COMPLEX†

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Wills, F.L., M^{c}Cubbin, W.D., & Kay, C.M. (1993) *Biochemistry 32*, 2321-2328.

INTRODUCTION

Calmodulin is a member of the EF-Hand superfamily originally described by Kretsinger (see review: Moncrief *et al.*, 1990) in reference to the helix-loop-helix motif used by members of this group to bind Ca^{2+}. Calmodulin is known to interact in a Ca^{2+} dependent fashion and regulate a number of other proteins including myosin light chain kinase, cyclic nucleotide phosphodiesterase, Ca^{2+}/calmodulin dependent protein kinase II, and phosphorylase kinase. A partial list of other proteins in this EF-Hand superfamily includes troponin C, calcineurin, oncomodulin, parvalbumin, S-100, myosin light chains, calpain (Moncrief *et al.*, 1990) and SMCaBP-11 (Mani & Kay, 1990). Since calponin had been described to interact with two other members of this group in addition to calmodulin, troponin C (Takahashi *et al.*, 1988b) and calpain (Tsunekawa *et al.*, 1989), it suggested there may be a common determinant among these proteins conferring upon them the ability to interact with calponin. In particular a goal of this investigation was to establish whether calponin specifically interacted with calmodulin, or if it was a non-specific interaction due to a reaction site on calponin that would optimally be used to bind a similar but separate protein.

In our pursuit of this question, the stoichiometry and strength of calponin's interaction with calmodulin has been characterized, and the dissociation constants compared with those of four other members of the EF-Hand family: Troponin C,

the Ca^{2+} binding subunit of the troponin complex which confers Ca^{2+} sensitivity upon cardiac and skeletal muscle contraction; S-100b, the β-β dimer form of the S-100 isoforms whose functions are not fully delineated; SMCaBP-11, a Ca^{2+} binding protein isolated from chicken gizzard which forms a dimer of 21 kDa; and parvalbumin, whose proposed function is as a relaxing factor of muscle by binding to Ca^{2+} as it is released from regulatory proteins following contraction. In addition to the fact that all of these proteins bind Ca^{2+}, all of them except parvalbumin expose hydrophobic patches upon Ca^{2+} binding. These hydrophobic patches are believed to be the site of interaction with many target proteins (Strynadka & James, 1989). Parvalbumin was thus included to test the necessity of the hydrophobic patch for interaction. A major finding was that this area does indeed seem to be required for interaction and it is not simply a complexation based upon the basic nature of calponin and the acidic nature of the Ca^{2+} binding proteins which enables interaction. Furthermore it would seem the interaction between calponin and calmodulin may be non-specific since we found there are other hydrophobic patch exposing proteins which interact with calponin with greater affinity.

RESULTS

Calponin-Calcium Interaction

There have been reports in the literature that calponin binds Ca^{2+} [Takahashi *et al.* (1988b), Winder & Walsh (1990b)]; however under our conditions

we were unable to find any signs of Ca^{2+} interaction. Calcium addition to calponin had no effect upon the circular dichroism spectrum indicating there was no change in secondary structure upon Ca^{2+} addition (see figure 4.1). Provencher-Glöckner analysis of the far UV circular dichroism spectrum of calponin indicated the protein is comprised of 41% α-helix, 17% β-sheet, 12% β-turn, and 29% random coil in the presence or absence of Ca^{2+}. In addition the UV difference spectrum (not shown) and the fluorescence of acrylodan calponin showed no change upon Ca^{2+} addition (see figure 4.2). To further explore the possibility of an interaction, Quin 2 which is a fluorescent indicator of calcium concentration, and equilibrium dialysis in the presence of $^{45}Ca^{2+}$, were performed, which indicated that there was no Ca^{2+} bound to calponin under the conditions employed. Thus, by all the criteria explored, we were unable to find any evidence that calponin interacts with Ca^{2+} which is supported by the fact that the published calponin sequence does not contain any EF-Hand-like regions (Takahashi & Nadal-Ginard, 1991).

Stoichiometry of the calponin-calmodulin complex

Analytical ultracentrifugation of the calponin calmodulin complex indicated molecular weights ranging from the molecular weights of the individual proteins to well above the calponin (32.3 kDa) and calmodulin (16.7 kDa) theoretical 1:1 complex weight of 49 kDa. Neither calponin nor calmodulin alone, either in the presence or absence of calcium, demonstrated molecular weights above their monomer values indicating there was no aggregation of either protein alone (see

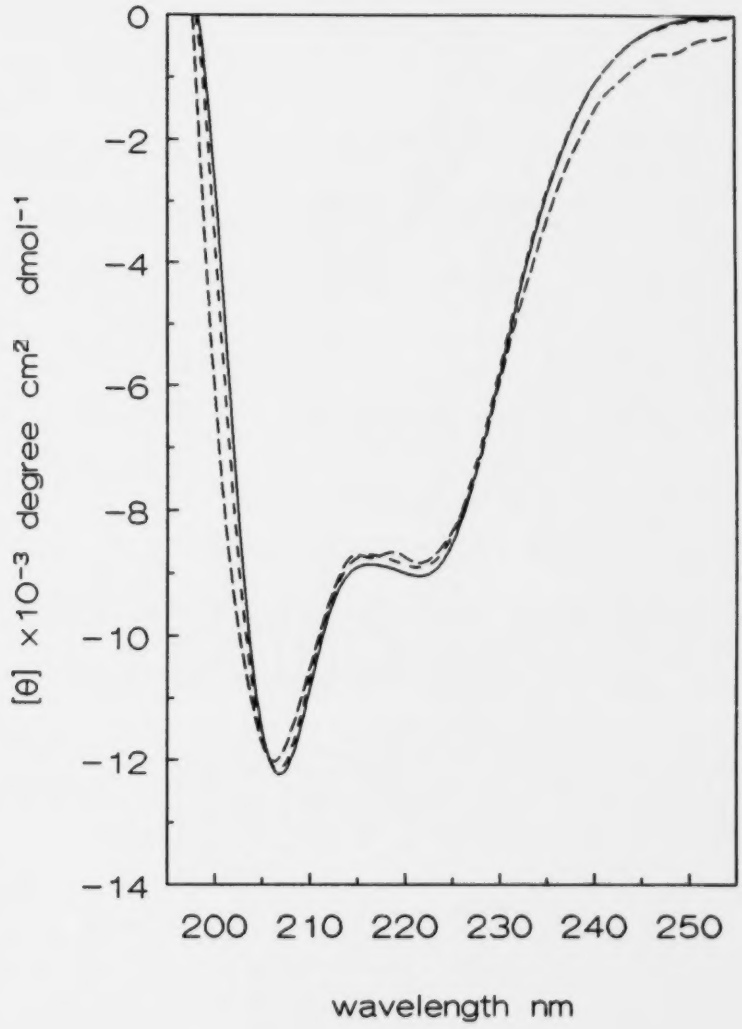

Figure 4.1: Far ultraviolet CD spectra of native calponin in 50 mM MOPS pH 7.15, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA (——), or 1 mM free Ca^{2+} (-----). Far UV CD spectrum of acrylodan labelled calponin (— —).

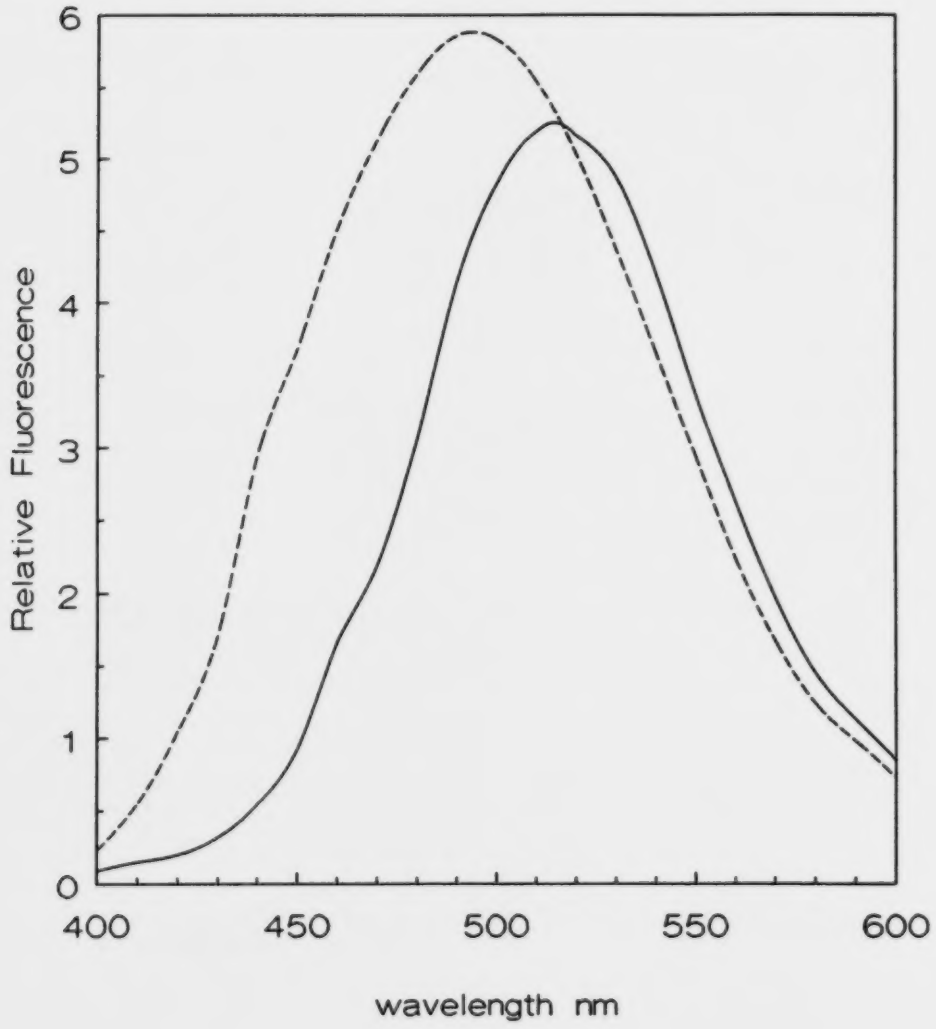

Figure 4.2: Fluorescence spectra of acrylodan calponin, acrylodan calponin + Ca^{2+}, acrylodan calponin + calmodulin (——), and acrylodan calponin + Ca^{2+}-calmodulin (-----).

figure 4.3). The slope of the lny vs. r^2 plot was used to determine the molecular weight, and deviation from the straight line near the meniscus indicates the presence of lower molecular weight species at this position in the cell. In the case of calponin which shows a molecular weight of 32.1 kDa, these lower molecular weight species are breakdown products which are produced over the 48 hour course of the experiment and can be seen by SDS-PAGE (data not shown). Calmodulin does not exhibit any deviation from linearity and shows a molecular weight of 17.2 kDa, well within experimental error of calmodulin's monomer weight of 16.7 kDa. Thus the high molecular weights for the mixture must be due to complex formation. In order to determine the stoichiometric amounts of calponin and calmodulin in the complex, varying ratios of calponin and calmodulin were mixed to see if excess of either protein favoured complex formation (see table 4.1). When calponin was in excess, a molecular weight of only 50 kDa was seen indicating no more than a 1:1 complex. However when excess calmodulin was present, a molecular weight of 63.3 kDa was observed across the bottom third of the cell indicating the nature of the complex to be 2 calmodulin molecules to 1 calponin (see figure 4.3C). The fact that the slope of the lny vs r^2 plot curved near the meniscus indicated not all the protein was complexed and suggested the interaction was not a strong one. When the experiment was carried out in the absence of Ca^{2+}, no molecular weights were observed above those of the constituent proteins indicating the interaction was dependent upon the presence of Ca^{2+}. In addition, a 3 calmodulin to 1 calponin mixture in the presence of Ca^{2+}

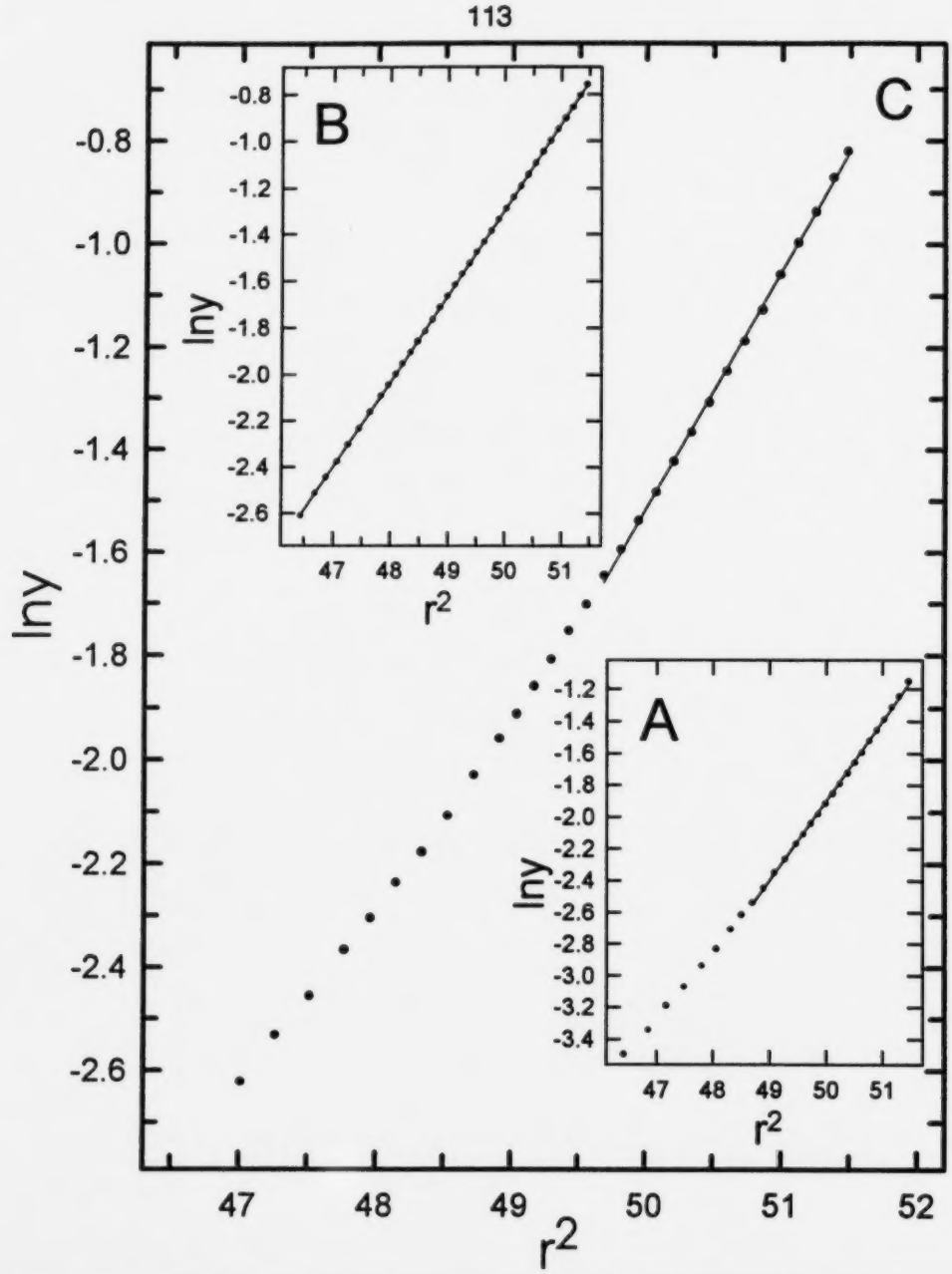

Figure 4.3: Representative plots of ln y vs. r^2. The slope of the line was determined from a least squares fit of the data points through which the line passes and is used to calculate the molecular weight. In all cases the fit to the data had a R value of >0.999. A: calponin, molecular weight 32.1 kDa; B: calmodulin, molecular weight 17.3 kDa; and C: a 2 mol calmodulin to 1 mol calponin ratio, molecular weight 63.3 kDa. The buffer used was 50 mM MOPS pH 7.15, 100 mM NaCl, 1 mM DTT, and 1 mM $CaCl_2$.

Table 4.1: Analytical Ultracentrifugation of the Calponin-Calmodulin Complex

Protein mol ratios	calcium	molecular weight observed
calponin	±	32.1 kDa
calmodulin	±	17.3 kDa
calponin + calmodulin	-	29.0 kDa
2 calponin + 1 calmodulin	+	50.8 kDa
1 calponin + 2 calmodulin	+	63.3 kDa
1 calponin + 3 calmodulin	+	63.4 kDa

was used to determine if higher molecular weights could be observed; however, the molecular weights across the bottom third of the centrifuge cell still remained at 63.4 kDa indicating this is truly the upper limit of complexation. This experiment was repeated with other calcium binding proteins to see if they formed the same type of complex and evidence indicates troponin C, S-100b, and SMCaBP-11, all of which expose hydrophobic patches upon Ca^{2+} binding, also form this complex of 2 mol of the relevant Ca^{2+} binding protein : 1 mol calponin in a Ca^{2+} dependent manner. In the case of S-100b and SMCaBP-11 which exist as dimers, molecular weight data indicated 2 mol dimer bound to 1 mol calponin. On the other hand, parvalbumin, which does not expose hydrophobic patches upon Ca^{2+} binding, showed no signs of binding to calponin as the molecular weights observed were representative of the constituent monomers.

Acrylodan labelling of calponin

Since the intrinsic tryptophan fluorescence of calponin did not respond in a significant manner to interaction with calmodulin, the fluorescent label acrylodan was used to label the exposed cysteine of calponin, thereby introducing a probe that is sensitive to ligand binding. DTNB titration of calponin indicated that although there is more than one cysteine per calponin molecule, 2 or 3 depending upon the isoform (Takahashi & Nadal-Ginard, 1991), when DTNB was reacted with calponin under non-denaturing conditions there was 1.3 mol cysteine per mol calponin which reacted immediately with DTNB, while it required more than 30

minutes for two mol cysteine per mol calponin to interact (see figure 4.4). This indicates that there is one cysteine in a highly exposed environment on the calponin, while the other cysteines are more buried. We have observed that the integrity of the other cysteines must remain intact by the following observations: reaction with DTNB of more than one cysteine per calponin leads to instability of the protein and precipitation; labelling of calponin under denaturing conditions with acrylodan leads to loss of secondary structure as detected by far UV circular dichroism; carboxymethylation of the cysteines under denaturing conditions also leads to loss of secondary structure; and maintenance of a reduced environment is very important for the function and structural integrity of calponin. Therefore since the non-denaturing conditions used for labelling with acrylodan lead to a calculated average of one acrylodan label per calponin molecule, it is likely that there is only one highly exposed cysteine which is labelled, while the others are more buried within the protein. This singly labelled calponin retains its secondary structure as seen by circular dichroism (see figure 4.1) and behaves like native calponin with regards to its biological activity in inhibiting actin activation of the myosin ATPase activity (data not shown). In order to determine if acrylodan labelled calponin interacted with calmodulin in an identical fashion to native calponin, fluorescence titrations of various ratios of labelled vs. unlabelled calponin with increasing amounts of calmodulin were performed. The resulting fluorescent curves are superimposable indicating the labelled and unlabelled calponin indeed

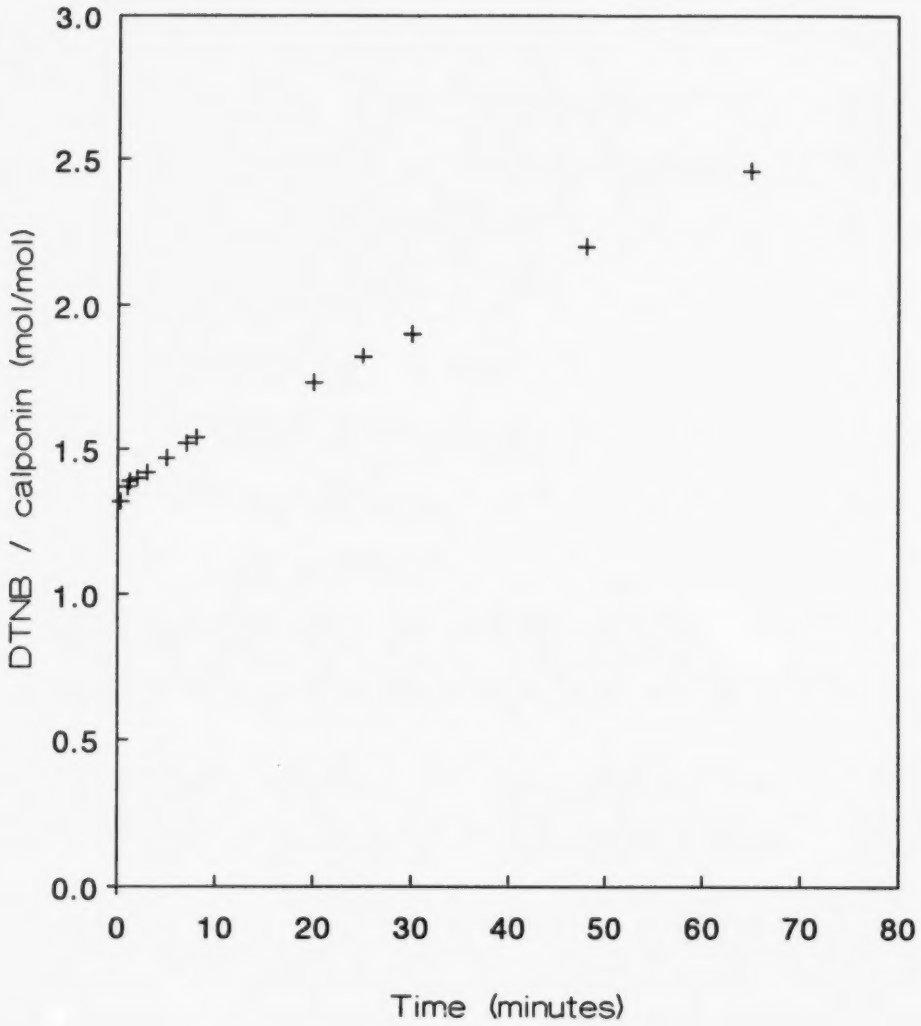

Figure 4.4: DTNB Titration of calponin in 50 mM MOPS pH 7.15, 100 mM NaCl, and 1 mM EGTA. The initial calponin concentration was 8 μM, and the reaction was quantitated by monitoring the increase in absorbance at 412 nm.

behave in a similar fashion and it is reasonable to use the acrylodan labelled calponin (see figure 4.5).

Fluorescence of the Calponin-Calmodulin Complex

The acrylodan probe on calponin is a very sensitive monitor of the interaction between calponin and the calcium binding proteins. The emission maximum of acrylodan bound to β-mercaptoethanol in the polar environment of water is 540 nm, while the very nonpolar environment of dioxane shifts the emission maximum to 435 nm (Prendergast, 1983). As shown in the fluorescence scan (figure 4.2) acrylodan calponin alone has an emission maximum at 515 nm which would indicate the acrylodan probe is in a polar, exposed environment. The position of this peak is unaffected by calcium or by calmodulin in the absence of calcium (see figure 4.2). However, when calmodulin is added in the presence of calcium, there is a shift in the emission peak to 495 nm indicating the probe has moved into a less polar, less exposed environment. This indicates that after the complexation, the hydrophobic fluorophore becomes buried either within the protein or within the calponin - calmodulin interface. In addition to this peak shift there is also an increase in the fluorescence of the acrylodan calponin and these two effects combine to produce the greatest change at 460 nm where the fluorescence, in the presence of saturating amounts of calcium-calmodulin, is 3.5 times the fluorescence of acrylodan calponin alone. This blue shift in emission maximum and increase in fluorescence also occurs in an analogous fashion with

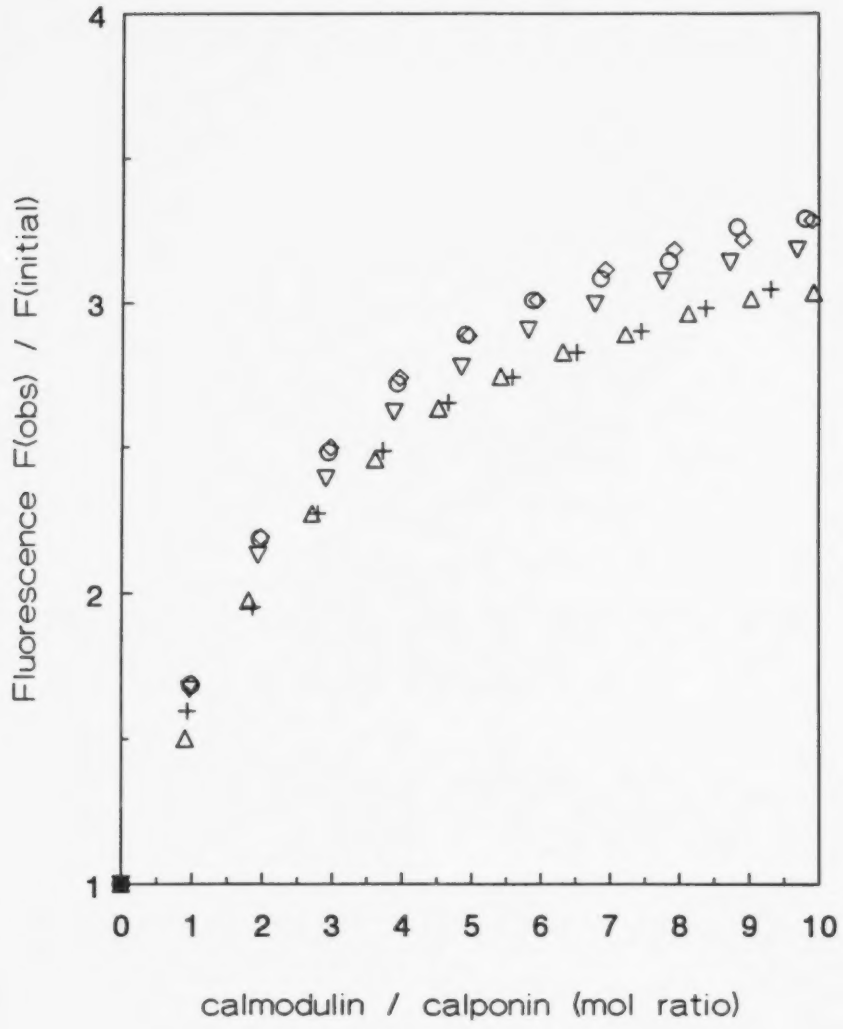

Figure 4.5: Titration of various ratios of acrylodan:native calponin with calmodulin. +, 12%; ▵, 22%; ○, 46%; ▿, 70%; ◇, 84% of total calponin is labelled with acrylodan. The buffer used was 50 mM MOPS pH 7.15, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 3 mM $CaCl_2$.

the hydrophobic-patch-exposing proteins troponin C, S-100b, and SMCaBP-11 in a calcium dependent fashion. On the other hand, parvalbumin did not cause any change in the fluorescence.

The large increase in fluorescence at 460 nm of acrylodan calponin can be monitored as the calponin is titrated with the calcium binding proteins in the presence of calcium (see figure 4.6). From these curves it is evident that troponin C has the weakest affinity for calponin of the proteins tested and causes the smallest (2 fold) increase in the fluorescence. Calmodulin has an intermediate affinity and causes a 3.5 fold increase in fluorescence but it takes approximately a 40 fold molar excess of calmodulin over calponin to do so. S-100b and SMCaBP-11 however show 4 fold increases in fluorescence requiring only a two molar ratio excess over calponin to do so with the highest affinities for calponin.

Based upon the stoichiometry determined with the analytical ultracentrifuge of two calcium binding proteins binding to one calponin to form a complex, and the fact that the calmodulin and troponin C fluorescence curves did not fit a 1:1 binding curve, the fluorescence data curves for the interaction between calponin and these other proteins can be curve fitted for a 2:1 interaction to determine binding constants (see table 4.2). The results clearly indicate that troponin C binds with the overall weakest affinity with Kd_1, the binding of the first troponin C to calponin, of $\leq .68\ \mu M$ and Kd_2, the binding of the second troponin C to calponin, of 4.5-8.5 μM. Calmodulin demonstrated an intermediate affinity with a Kd_1 of $\leq .22\ \mu M$ and a Kd_2 of 2.5-3.4 μM. S-100b and SMCaBP-11 demonstrated higher

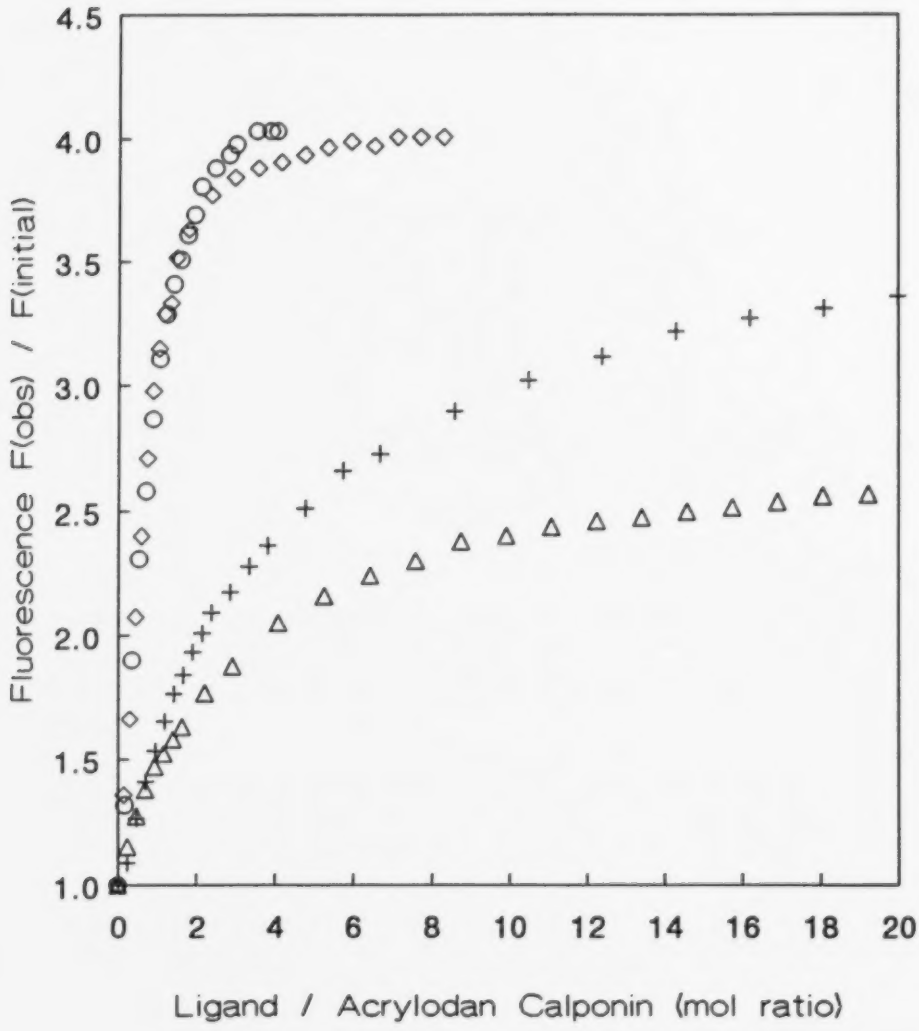

Figure 4.6: Fluorescence titration of calponin with Ca^{2+} binding proteins in the presence of Ca^{2+}. (△), TNC; (+), calmodulin; (◇), S-100b; and (○) is SMCaBP-11. Mol ratios for S-100b and SMCaBP-11 are calculated for the dimer.

Table 4.2: Dissociation Constants for Calponin-Ligand Complexes

LIGAND	Kd_1 (μM)[a]	F_1[b]	Kd_2 (μM)[a]	F_2[b]	F_{obs}/F_o
TNC	≤.68	.46±.05	4.5-8.5	.54±.09	2
Calmodulin	≤.22	.27±.07	2.5-3.4	.73±.08	3.5
SMCaBP-11	≤.13	.94±.1	.18-1.60	.06±.15	4
S-100b	≤.03	.83±.03	.36-.77	.17±.04	4
Parvalbumin	No Interaction				

[a] The range shown represents the mean ± the standard deviation.
[b] F_1 & F_2 represent the fractional fluorescence change observed for each ligand binding.

affinities, with SMCaBP-11 having a Kd_1 of ≤.13 μM and a Kd_2 of .18-1.6 μM while S-100b exhibited a Kd_1 of ≤.03 μM and a Kd_2 of .36-.77 μM. It is evident that these latter two proteins show considerably higher affinity for calponin than calmodulin does, and cause a larger increase in the quantum yield of the probe (Fobs/Fo in table 4.2) upon complexation. In addition, the first binding of SMCaBP-11 or S-100b to calponin causes a much greater increase in fluorescence than the first binding of troponin C or calmodulin.

Calcium titration of the calponin-calmodulin complex

The interaction between calponin and calmodulin is dependent upon calmodulin binding to Ca^{2+} as has been published previously (Takahashi *et al.*, 1986) and as demonstrated by the lack of an effect of calmodulin on the fluorescence spectrum of calponin in the absence of Ca^{2+} (see figure 4.2). The dependence of complex formation upon Ca^{2+} can be demonstrated by titration by monitoring the increase in fluorescence as the complex forms (see figure 4.7). From this titration 50% of the fluorescence change occurs with a pCa^{2+} of 5.9 which is very close to the pCa^{2+} of the low affinity sites predicted for calmodulin of 5.7, found in modelling studies by Wang (1985). This suggests calponin binding to calmodulin is dependent upon a change occurring in calmodulin upon calcium filling the low affinity sites, and does not respond to calcium filling of the high affinity sites which is predicted to occur at a pCa^{2+} of 6.7. Preliminary experiments

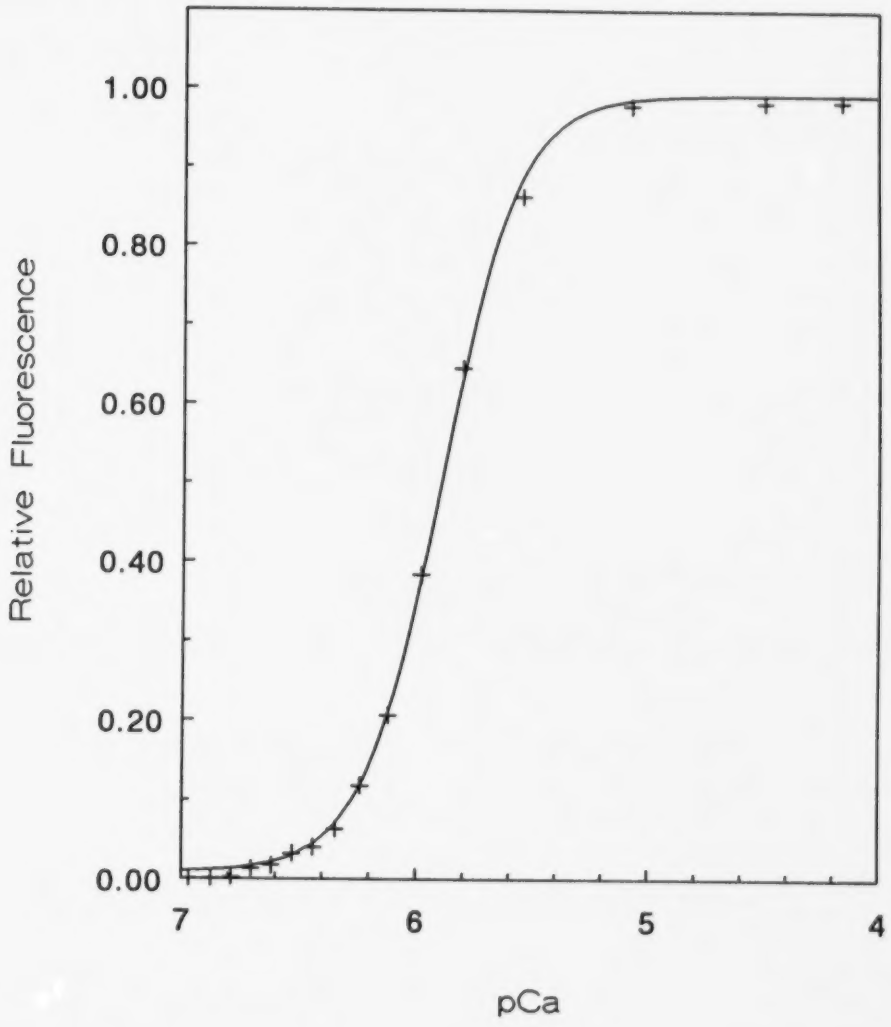

Figure 4.7: Fluorescence titration of the calponin-calmodulin complex with Ca^{2+} (+). The data was curve fitted to a sigmoidal transition equation(——).

indicated troponin C also required binding of Ca^{2+} to the low affinity sites for interaction with calponin (data not shown).

Far UV CD of the calponin-calmodulin complex

In order to determine whether secondary structural changes occur upon complexation of calponin with calmodulin, the far UV circular dichroism spectra of the two proteins in the presence of calcium were determined and then a theoretical curve of the two proteins together was calculated assuming no structural changes occur. This theoretical curve is then compared to the observed curve for the complex to see if any changes actually occurred upon complexation (see figure 4.8). This was done both in the presence and absence of Ca^{2+}. In the absence of Ca^{2+} the difference in ellipticity between the observed and calculated spectra for the two proteins was 440 degrees at 222nm which is just out with experimental error for this technique. However in the presence of Ca^{2+} there is 1450 degree decrease in negative ellipticity at 222nm in the observed spectrum vs. the theoretical curve indicating a change in secondary stucture in one or both components occurred upon complexation. Provencher-Glöckner secondary structure prediction analysis of both the theoretical and observed spectra was performed to determine the nature of the secondary structure changes. The observed spectra for the complex had 25% α-helix which indicates a 5% decrease occurred, and 28% β-sheet which indicates an 8% increase occurred upon complexation. Results from the analytical ultracentrifuge data indicate that when

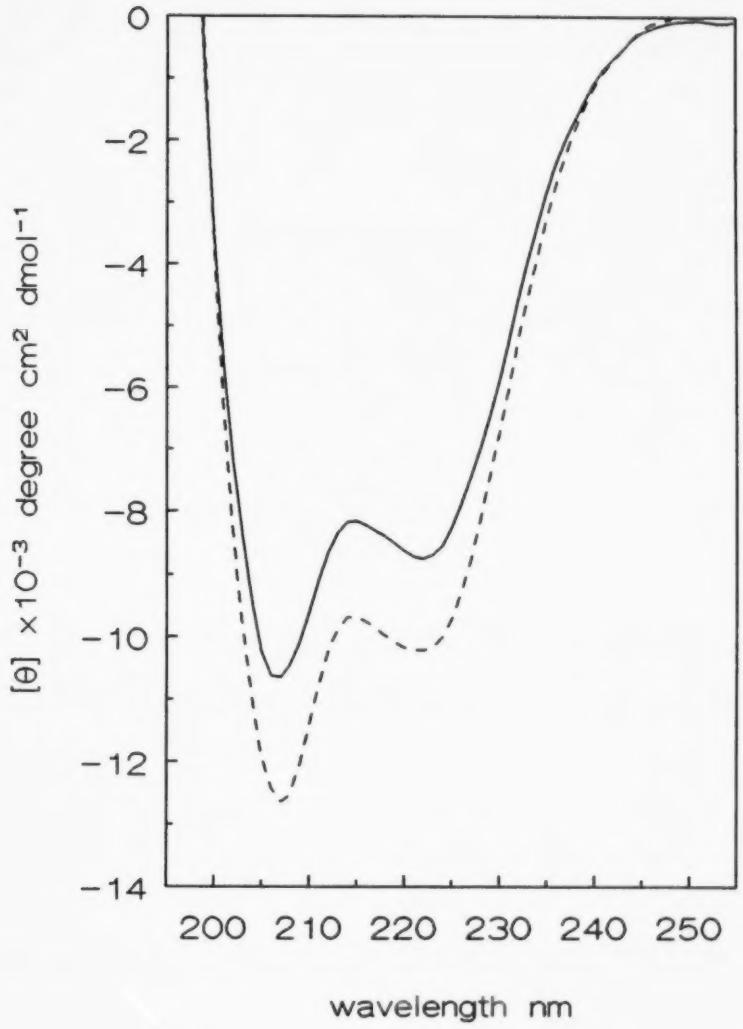

Figure 4.8: Far ultraviolet CD spectra of the calponin-calmodulin complex in the presence of Ca^{2+} (——), and the calculated theoretical curve for the two proteins assuming no interaction (-----).

calponin and calmodulin are mixed not 100% of the protein forms a complex as evidenced by the presence of lower molecular weight species at the top half of the cell (see figure 4.3C). Therefore the changes observed in the CD spectra are only an indication that secondary structural changes occur, and the spectrum observed does not represent the pure complex, but rather a mixture of complexed and free protein.

DISCUSSION

Calponin was originally described as a calcium, calmodulin, and actin binding troponin T-like protein [Takahashi *et al.*(1988b), Winder & Walsh (1990b)]. In view of this initial suggestion that calponin bound calcium, it was decided to investigate the relationship more closely. The studies reported herein on the physical properties of calponin were unable to demonstrate any evidence of calponin binding to calcium. This is not surprising since it has previously been reported that Ca^{2+} has no effect upon ATPase inhibition by calponin (Winder & Walsh, 1990a), or upon binding to actin or tropomyosin (Takahashi *et al.*, 1986). While calponin does not bind Ca^{2+} directly, it interacts in a calcium dependent fashion with proteins which expose a hydrophobic patch upon themselves binding Ca^{2+}. It has been previously reported that calponin is a substrate for the calcium dependent protease calpain which is known to digest calmodulin binding proteins (Tsunekawa *et al.*, 1989). This, together with the fact that calponin will bind both calmodulin

and troponin C (Takahashi *et al.*, 1986), suggested that there may be a common determinant among these proteins which conferred their ability to interact with calponin. Of the group tested in this study troponin C, S-100b, calmodulin, and SMCaBP-11, all expose these hydrophobic patches in a Ca^{2+} dependent fashion as shown by TNS titrations of these proteins [Tanaka & Hidaka (1981), Mani & Kay (1990)]. TNS interacts with hydrophobic regions resulting in a dramatic increase in quantum yield of the TNS. We have shown that all these hydrophobic patch exposing proteins interact with calponin in a calcium dependent manner. Parvalbumin, conversely, binds calcium but does not expose these hydrophobic patches which are postulated to provide the interface for interaction with target proteins (Strynadka & James, 1989). In agreement with our theory that it is these hydrophobic patches with which calponin is interacting, calponin does not bind to parvalbumin. The interaction between calponin and calmodulin was shown to be 50% complete at a pCa^{2+} of 5.9 which coincides with the predicted strength of calmodulin's low affinity sites for Ca^{2+} (Wang 1985). Tanaka & Hidaka (1980) showed that the dramatic increase in exposed hydrophobic residues in calmodulin occurred with a pCa^{2+} of 5.5 which also agrees with the amount of Ca^{2+} required for calponin-calmodulin complexation. Calmodulin exhibits stepwise changes in structure with the number of mol Ca^{2+} bound, and the changes in calmodulin at these various Ca^{2+} levels can be used to modulate various processes within the cell (Klee *et al.*, 1986). It has been shown that calmodulin interacting proteins may variously depend upon Ca^{2+} binding to the high affinity sites alone for activation,

for example phosphorylase kinase and plasma membrane ATPase, or upon Ca^{2+} binding to both the high and low affinity sites, as with cyclic nucleotide phosphodiesterase and myosin light chain kinase. Thus different levels of Ca^{2+} filling are required for various functions and calponin requires all 4 Ca^{2+} sites to be filled for interaction. What is surprising however is that proteins which require Ca^{2+} binding to the low affinity sites for complexation with calmodulin, generally demonstrate an increased Ca^{2+} affinity in the complex than calmodulin does alone, i.e. the low affinity sites fill with Ca^{2+} at a lower concentration of free Ca^{2+} when the receptor protein is present than in its absence. This increase in affinity may be as much as two orders of magnitude (Klee, 1988). That calmodulin, while interacting with calponin, does not display this increased affinity for Ca^{2+} does indicate the free energy of interaction between these two proteins is not strong enough to affect the Ca^{2+} binding affinity and that this interaction may not be biologically significant.

The nature of the complex between calponin and calmodulin was probed through the use of circular dichroism spectroscopy and analytical ultracentrifugation. From the far UV circular dichroism spectra it was found that there was a change in secondary structure resulting in decreased α-helix and increased β-sheet as complexation occurs compared to the two proteins individually. These changes do not occur in the absence of Ca^{2+}, once again emphasizing the Ca^{2+} sensitivity of the complex. Analytical ultracentrifuge experiments indicated a complex between calponin and calmodulin was being

formed which had a mass well above the theoretical weight for a 1:1 complex. Molecular weights obtained in the analytical ultracentrifuge indicate that in the presence of excess amounts of calponin a 1:1 complex between calmodulin and calponin is formed with an observed molecular weight of 50.8 kDa. However in the presence of excess calmodulin a molecular weight of 63.3 kDa was seen. This suggests the nature of the complex is two calmodulin molecules to one calponin, producing a complex of 66 kDa in the presence of calcium. However, when there is excess calponin present, the binding of the first calmodulin is stronger than the binding of the second calmodulin such that each calponin having one calmodulin bound is energetically more favourable than 50% of the calponin molecules having two calmodulins bound, with the result that only a 1:1 complex is observed. It has previously been observed in gel filtration experiments that an average of 1.2 molecules of calmodulin were eluting with each molecule of calponin suggesting that at least two calmodulins could bind per molecule of calponin (Takahashi *et al.*, 1986). Our analytical ultracentrifuge data indicates that even a three fold excess of calmodulin over calponin yields the 66 kDa molecular weight species indicating the nature of the complex is two calmodulins per calponin. Troponin C, S-100b and SMCaBP-11 also appear to interact with calponin in the same ratio, suggesting that all these proteins which are able to expose hydrophobic patches in the presence of Ca^{2+}, bind with the stoichiometry of two Ca^{2+} binding proteins per calponin molecule. In the case of SMCCaBP-11 and S-100b, which are functional as dimers, two mols of dimer are able to interact with each mol of calponin. Since

parvalbumin did not show any sign of interaction with calponin either in the presence or absence of Ca^{2+}, the reaction between these proteins and calponin is not simply due to the acidic nature of the calcium binding proteins and the basic nature of calponin. While parvalbumin is similar to the other proteins in the EF-Hand superfamily in being an acidic binding protein which binds Ca^{2+} with EF Hand like structures it does not expose hydrophobic patches upon Ca^{2+} binding and this seems to be the determining factor in the ability to bind calponin. The lack of interaction with parvalbumin underlines the importance of hydrophobic patches for calponin interaction.

The hydrophobic patches exposed upon calcium binding by calmodulin and troponin C have been implicated in the interaction with their target proteins (LaPorte *et al.*, 1980). It is believed that in order to interact, presentation of both hydrophobic and basic determinants is necessary, and some target proteins may form an amphipathic α-helix which is able to bind to the hydrophobic patches (Cachia *et al.*, 1986). Some degree of flexibility in the interaction of target proteins with non specific hydrophobic patches has already been demonstrated (Cachia *et al.*, 1985) and from this study it is evident calponin demonstrates this flexibility in binding to a variety of hydrophobic patch proteins, albeit with varying affinities.

Much controversy has surrounded the question as to whether the caldesmon-calmodulin interaction is strong enough that caldesmon would bind to calmodulin *in vivo* due to the fact that calmodulin binding to other target proteins, for example myosin light chain kinase and calcineurin, demonstrate Kd's in the low

nanomolar range compared to a Kd of 250nM for caldesmon (Mills *et al.*, 1988). Also the large mol ratios of calmodulin to caldesmon required to reverse caldesmon's inhibition of the actomyosin ATPase would argue against such a regulation being feasible (Marston, 1990). These same arguments apply even more so to the issue of calponin-calmodulin interaction in the cell. The present study would argue against the calponin-calmodulin interaction being biologically important as not only is the Kd for this interaction too weak, but also two other proteins, S-100b and SMCaBP-11 have demonstrated much greater affinities. In addition two separate groups have shown that as much as 10 mol ratios of calmodulin to calponin are required to reverse its inhibition [Abe *et al.* (1990) and Makuch *et al.* (1991)]. Thus it would appear the calmodulin-calponin interaction, like the calponin-troponin C interaction, is non-specific. With reference to S-100b and SMCaBP-11, the SMCaBP-11 is isolated from smooth muscle cells and appears to be a likely candidate for interaction with calponin in a biologically significant manner. S-100 was originally thought to be primarily a nervous tissue protein as it was derived from bovine brain; however varying isoforms of S-100 have now been reported in a variety of cell types including melanocytes, Langerhans cells, slow twitch skeletal muscles, heart, and kidney tissue (Donato, 1991). Although S-100 has not been specifically isolated from smooth muscle tissue, it cannot be ruled out that calponin and S-100b or an S-100b like protein could be present in the same tissue. Pritchard & Marston (1991) have attempted to isolate an S-100-like protein with regulatory abilities from smooth muscle;

however to date this effort has been unsuccessful.

The ability of calponin to interact in a non specific fashion with these Ca^{2+} binding proteins can be related to its function in inhibiting the ATPase of actomyosin. Horiuchi and Chacko (1991) showed that while HMM bound to actin and the ATPase was active in the absence of calponin, increasing ratios of calponin/actin not only caused a drop in ATPase activity but also caused a concomitant decrease in the HMM bound to actin. Since it is known that calponin binds to actin (Takahashi *et al.*, 1986) it seems that actin is able to bind either calponin or HMM but their interaction is mutually exclusive. This suggests the method by which calponin inhibits the ATPase is by binding to a site on actin such that myosin is no longer able to bind to actin. Makuch *et al.* (1991) showed that a 10 fold excess of Ca^{2+}-calmodulin over calponin can displace calponin from actin in ultracentrifugation experiments and also restore ATPase activity. This would be directly analogous to the findings of Van Eyk *et al.* (1991) in the skeletal muscle case. These researchers found that the troponin I inhibitory peptide can bind to either actin in the absence of Ca^{2+} which confers inhibition of ATPase activity and contraction, or to troponin C in the presence of Ca^{2+} which leaves actin free to interact with myosin allowing ATPase activity and contraction to occur. Van Eyk *et al.* (1991) showed that actin and troponin C compete for the same site on troponin I, indicating a similar determinant on troponin I must be recognized by both these proteins. Thus it would appear the myosin-actin-troponin I-troponin C system in skeletal muscle is a reasonable model for myosin-actin-calponin-

calmodulin behaviour in smooth muscle. It, therefore, is not unreasonable that calponin's ability to bind to actin with high affinity (Winder *et al.*, 1991) would confer upon it the ability to interact with proteins such as calmodulin, S-100b, and SMCaBP-11 which are similar to troponin C. It will be of great interest to pursue these relationships and determine which proteins are biologically important in their interactions with calponin.

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Chapter 5

SMOOTH MUSCLE CALPONIN-CALTROPIN INTERACTION: EFFECT ON BIOLOGICAL ACTIVITY AND STABILITY OF CALPONIN†

†A version of this chapter has been previously published:
Wills, F.L., M^{c}Cubbin, W.D., & Kay, C.M. (1994) *Biochemistry 33*, 5562-5569.

INTRODUCTION

Originally calmodulin was identified as a possible calcium sensitizing agent because it bound to calponin in a calcium sensitive fashion. It turned out, however, that the mol ratio of calmodulin to calponin required to reverse calponin's inhibition is too great to suggest that this interaction could be of biological importance (Abe *et al.*, 1990, Makuch *et al.*, 1991). More recently our laboratory has shown that calponin will interact with a variety of calcium binding proteins which expose hydrophobic patches upon interaction with calcium. Among the proteins tested were two which displayed higher affinities for calponin than calmodulin did: S-100b and caltropin.(Wills *et al.*, 1993) Since caltropin is isolated from smooth muscle tissue it is a bona fide candidate as a regulator of calponin inhibition. Caltropin was first described by Mani & Kay (1990). This smooth muscle protein binds 2 mols calcium per mol monomer, and exposes a hydrophobic patch upon binding calcium. It has been shown, using analytical ultracentrifugation, that a molecular mass of 21 000 Da, representing a dimer of caltropin, is observed under native conditions. Upon binding calcium, caltropin undergoes structural changes as reflected by circular dichroism, UV difference spectroscopy, and fluorescence. Therefore this protein has the requirements to function as a calcium sensitive protein in smooth muscle (Mani & Kay, 1990).

The characterization of the calponin-caltropin complex is of great interest

particularly if it is a regulatory complex of smooth muscle. In this study, hydrodynamic and spectroscopic techniques have been used to determine the properties of the complex, which included stoichiometry of the protein constituents in the complex, its stability to guanidine and temperature, as well as its calcium sensitivity, and secondary structural changes occurring upon interaction of the two relevant proteins.

RESULTS & DISCUSSION

Analytical Ultracentrifugation of the calponin-caltropin complex.

In order to determine the nature of the complex between caltropin and calponin, analytical ultracentrifugation was performed using varying ratios of the two proteins. Earlier studies had shown that calmodulin combines with calponin in a ratio of 2 mols calmodulin : 1 mol calponin. This was demonstrated by the finding that in the presence of excess calponin to calmodulin, a molecular mass representing a 1:1 interaction was observed, while in the presence of 2 or 3 times the mol ratio of calmodulin to calponin, a molecular mass representative of a 2 calmodulin : 1 calponin complex was demonstrated (Wills *et al.*, 1993). It was also shown in this earlier paper that the formation of the complex required the presence of calcium. In addition, experiments have been performed on troponin C and S-100b, which also form a complex of 2 calcium binding proteins : 1 calponin in a calcium dependent fashion (data not shown). In the present study, the molecular

mass of the caltropin-calponin complex also varies in a calcium dependent fashion, and depends as well upon the mol ratio of the interacting proteins. The nature of complex formation was established by analytical ultracentrifugation in the presence and absence of calcium. The molecular mass observed reflects the weighted contribution of all species in the cell, and since there is a heterogeneous population present including the 2 initial constituents plus any complexes formed, a range of molecular mass is obtained in any individual experiment. In the representative experiment shown in figure 5.1, the proteins were combined in a ratio of 2 mols caltropin dimer to 1 mol calponin at an initial loading concentration of 0.7 mg/ml. The slope of the lny vs. r^2 plot was used to calculate the molecular mass, and deviation from the straight line near the meniscus indicates the presence of lower molecular mass species at this position in the cell due to uncomplexed protein. In the presence of calcium, a molecular mass of 70668 Da was obtained, which is within experimental error of the combined mass of one calponin (32333 Da), and two caltropins (21000 Da/unit) for a total of 74333 Da. If a third caltropin was able to bind to this complex the molecular mass would increase to 95333 Da, which did not occur in this experiment. In the absence of calcium, a molecular mass range of 20.4-27.1 kDa (data not shown), representative of the constitutive proteins was observed, demonstrating that the formation of the complex was dependent upon the presence of calcium. In order to be certain the observed molecular mass, which indicated the complex formed was greater than 1 calponin : 1 caltropin, did not arise from 2 calponin : 1 caltropin, the proteins

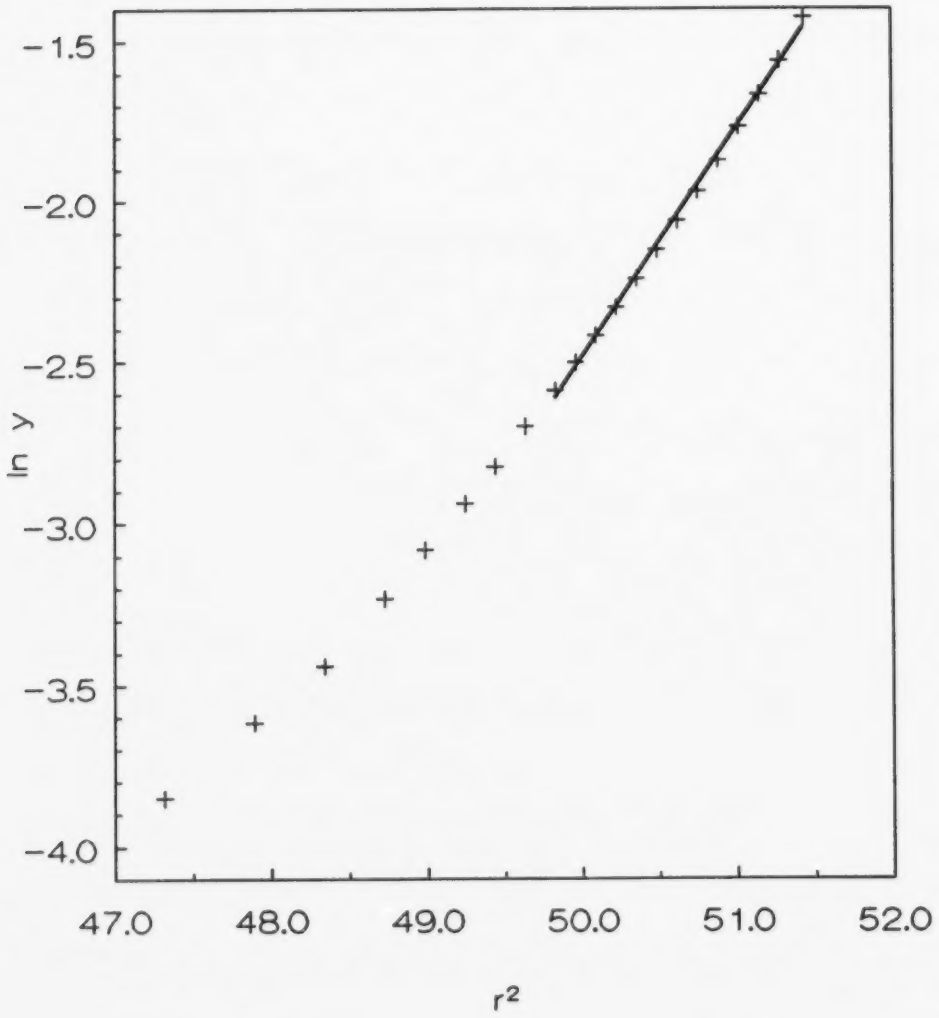

Figure 5.1: Representative plot of ln$y$ vs. r^2 for the calponin-caltropin complex. The slope of the line was determined from a least-squares fit of the data points through which the line passes and is used to calculate the molecular mass. A mol ratio of 2 caltropin dimers to 1 calponin was used at an initial concentration of 0.7 mg/ml, the speed was 13000 rpm, and the cell was maintained at 20°C.

were combined in a ratio of excess calponin to caltropin. A range of molecular mass of 29.3-48.6 kDa (data not shown) was observed, representative of a 1:1 interaction together with uncomplexed protein, indicating the higher molecular mass observed for the complex in figure 5.1 only occurs with excess caltropin and is truly indicative of a complex of two caltropins to one calponin. Previously it had been shown in the analytical ultracentrifuge that calponin alone, in the presence or absence of calcium, has a molecular mass of 32.1 kDa (Wills *et al.*, 1993), and caltropin, in the presence or absence of calcium, has a molecular mass of 21.0 kDa (Mani & Kay, 1990). Therefore the higher molecular mass observed in this experiment is indeed due to complex formation and not simply the result of aggregation of the constituent proteins. Since calponin does not interact with calcium, as demonstrated previously (Wills *et al.*, 1993), complex formation is dependent upon a structural change that has been shown to occur in caltropin when this protein binds calcium (Mani & Kay, 1990). The complex is formed in the same fashion as the calponin-calmodulin complex in that it consists of two mols calcium binding protein to one mol calponin, and its formation is calcium dependent. However, in this case, the functional unit of the calcium binding protein, caltropin, is a dimer.

Fluoresence Spectroscopy.

Since intrinsic static tryptophan fluorescence of calponin is not sensitive to interaction with calcium binding proteins (Wills *et al.*, 1993), acrylodan was used

to label calponin. The acrylodan fluorescence of calponin is sensitive to interactions of calponin with calcium binding proteins, and is therefore a useful probe to monitor complex formation. We have previously shown that reaction of calponin with the cysteine specific probe acrylodan under native conditions resulted in a singly labelled calponin that binds calcium binding proteins in the same fashion as native calponin as shown by fluorescence titrations, maintains the same secondary structure as shown by circular dichroism, and behaves similarly in the ATPase biological assay as native calponin (Wills *et al.*, 1993). Acrylodan calponin, when excited at 388 nm, has an emission maximum at 505 nm (figure 5.2). Neither the fluorescence intensity, nor the peak wavelength is affected by the presence of caltropin/EGTA, or the presence of Ca^{2+}. When caltropin is added in the presence of Ca^{2+}, however, there is a 35 nm blue shift in the peak of the spectrum from 505 nm to 470 nm. Since the greatest change in fluorescence intensity occurs at 460 nm, this wavelength was chosen for fluorescence titrations. Caltropin increases the fluorescence of acrylodan calponin 4½ times at this wavelength. These effects are greater than those previously shown for calmodulin which causes a 25 nm blue shift and a 3½ times increase in the fluorescence intensity of acrylodan calponin at 460 nm (Wills *et al.*, 1993). These results are indicative of the probe moving into a more non-polar environment when calponin interacts with caltropin, perhaps becoming more buried in the protein as the complex forms (Prendergast *et al.*, 1983), a response which allows for a sensitive measure of complex formation. Titration of acrylodan calponin with caltropin in the

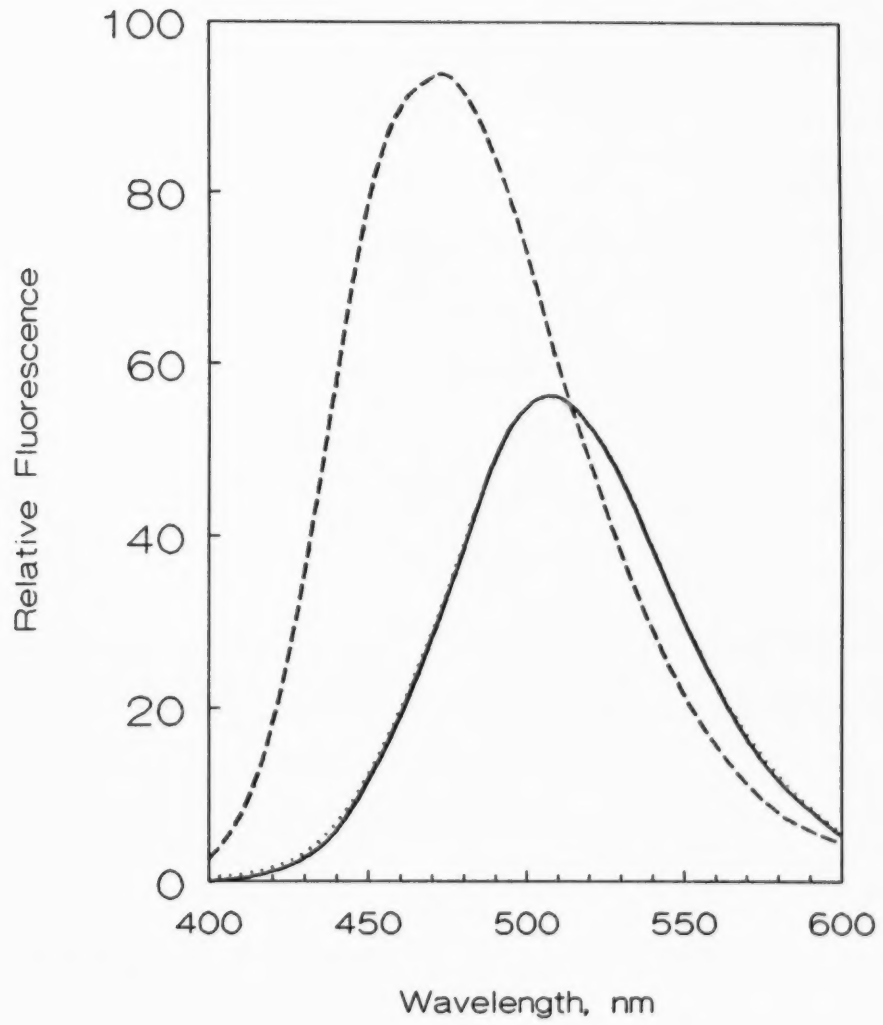

Figure 5.2: Fluorescence spectra of acrylodan labelled calponin & acrylodan calponin in the presence of Ca^{2+}(——), acrylodan calponin in the presence of caltropin and EGTA (• • • •), and acrylodan calponin in the presence of caltropin and Ca^{2+} (----). The wavelength of excitation is 388 nm.

presence of Ca^{2+} (figure 5.3) shows that the response is saturated with 4 mol ratios of caltropin. This curve can be fitted using the model of 2 mols calcium binding protein binding to each mol of calponin, determined in the sedimentation equilibrium experiments, to calculate dissociation constants. A Kd_1 for the first mol of caltropin binding to calponin can be calculated of $\leq 0.13\ \mu M$, and this interaction accounts for 94% of the fluorescence change. Kd_2, calculated for the second mol of caltropin interacting with calponin, is 0.18-1.6 μM and produces 6% of the fluorescence change (Wills *et al.*, 1993). The ranges quoted represent the Kd ± the standard deviation. This affinity is significantly higher than the values found when the calmodulin titration is fitted to this model of $\leq .22\ \mu M$ for Kd_1 and 2.5-3.4 μM for Kd_2 (Wills *et al.*, 1993), and is reflected in the fact that it takes 40 mol ratios of calmodulin over calponin to saturate the fluorescence effect due to the weaker binding affinities. Thus caltropin is a more suitable candidate for regulating calponin's inhibitory behavior than is calmodulin.

Location of acrylodan label on calponin.

The location of this acrylodan label has now been identified by performing a complete tryptic digest of the acrylodan labelled calponin and purifying the peptide labelled with acrylodan by monitoring the fluorescence off a reversed phase HPLC column. The acrylodan containing peptide was then sequenced and the sequence found was -VYDPKY which is located uniquely at residues 267-272. Cysteine 273 in chicken gizzard calponin was identified as the labelled residue.

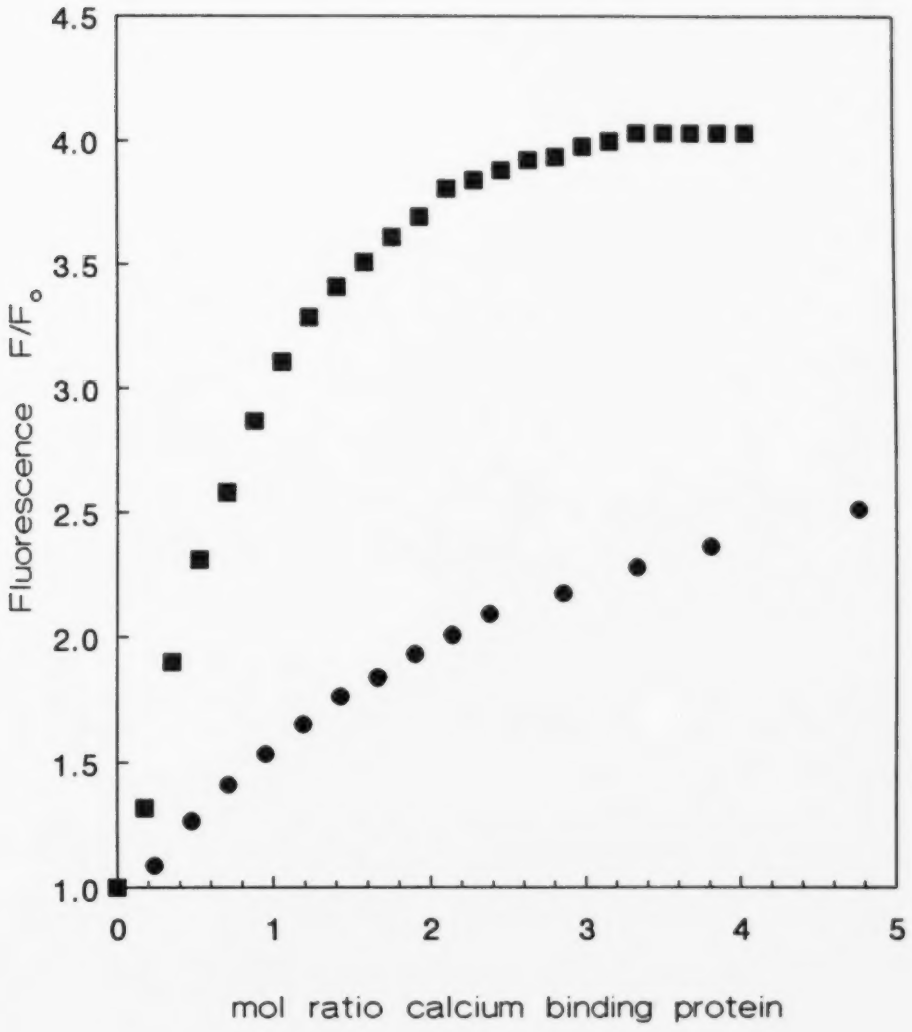

Figure 5.3: Titration of acrylodan calponin fluorescence at 460 nm, in the presence of Ca^{2+}, with increasing concentrations of caltropin(■), and calmodulin (●). Mol ratios for caltropin are calculated for the dimer. F is the observed fluorescence, while F_o is the initial fluorescence intensity.

Mezgueldi *et al.* (1992) have mapped the domains of interaction of calponin to actin, tropomyosin, and calmodulin through the use of affinity chromotography of proteolytic fragments. They found that calmodulin, tropomyosin and actin binding abilities were all maintained in a fragment constituting residues 1-184, and further, fragment 1-145 was able to bind calmodulin and tropomyosin but not actin. Whether 1-145 bound one or two mols of calmodulin was not determined. Nakamura *et al.* (1993) used the same method to determine that the carboxyl terminal 12 kDa comprising residues 183-292 did not bind actin, calmodulin or tropomyosin. Cysteine 273 is not near the actin, calmodulin, or tropomyosin binding sites as elucidated by these mapping experiments which indicates that the carboxyl terminal either is structurally affected by binding of these proteins or folds near the amino terminal region in the three dimensional structure. Cysteine 273 is the most carboxy-terminal of the cysteine residues in chicken gizzard calponin. Strasser *et al.* (1993) have sequenced mammalian isoforms of calponin from mouse and pig. They have shown that this cysteine is the last conserved residue between the various isoforms before a hyper-variable region at the carboxyl-terminal begins in mammalian calponin isoforms.

Calcium Titration of the calponin and caltropin complex.

The acrylodan fluorescence of calponin increases in the presence of caltropin/calcium whereas this does not occur in the presence of caltropin/EGTA. Therefore complex formation can be monitored with increasing calcium levels in

order to determine the concentration of calcium needed for interaction (figure 5.4). The midpoint of this titration is at a pCa of 4.04. Caltropin has been shown to bind 2 mols calcium per mol monomer, and a pCa of 4.04 corresponds to the low affinity calcium binding sites of caltropin of 4.09 as determined by Mani & Kay (1990). The high affinity sites of caltropin bind calcium at a pCa of 6.69. At this calcium concentration there is no evidence of complex formation as judged by fluorescence change. This finding indicates that when the smooth muscle cell is at rest and the calcium levels are approximately $1x10^{-7}$ M (Bagshaw, 1993) there would be no interaction; the complex would form when calcium levels in the cell are elevated during contraction to $1x10^{-5}$ M and could therefore constitute a regulatory complex for smooth muscle contraction. The difference between observed calcium levels present during excitation ($1x10^{-5}$ M), and those observed for complex interaction ($9x10^{-5}$ M), could easily be accounted for by the difference in environment *in vitro* compared to the natural, more stable environment of the thin filament.

Circular dichroism of the complex

In order to determine if secondary structural changes occur upon interaction, far ultraviolet circular dichroic spectra of the calponin-caltropin complex were determined in the presence and absence of calcium (figure 5.5). The proteins were mixed as 2 mol ratios of caltropin dimer per mol of calponin since this is the stoichiometry of complex formation. In addition, theoretical spectra for

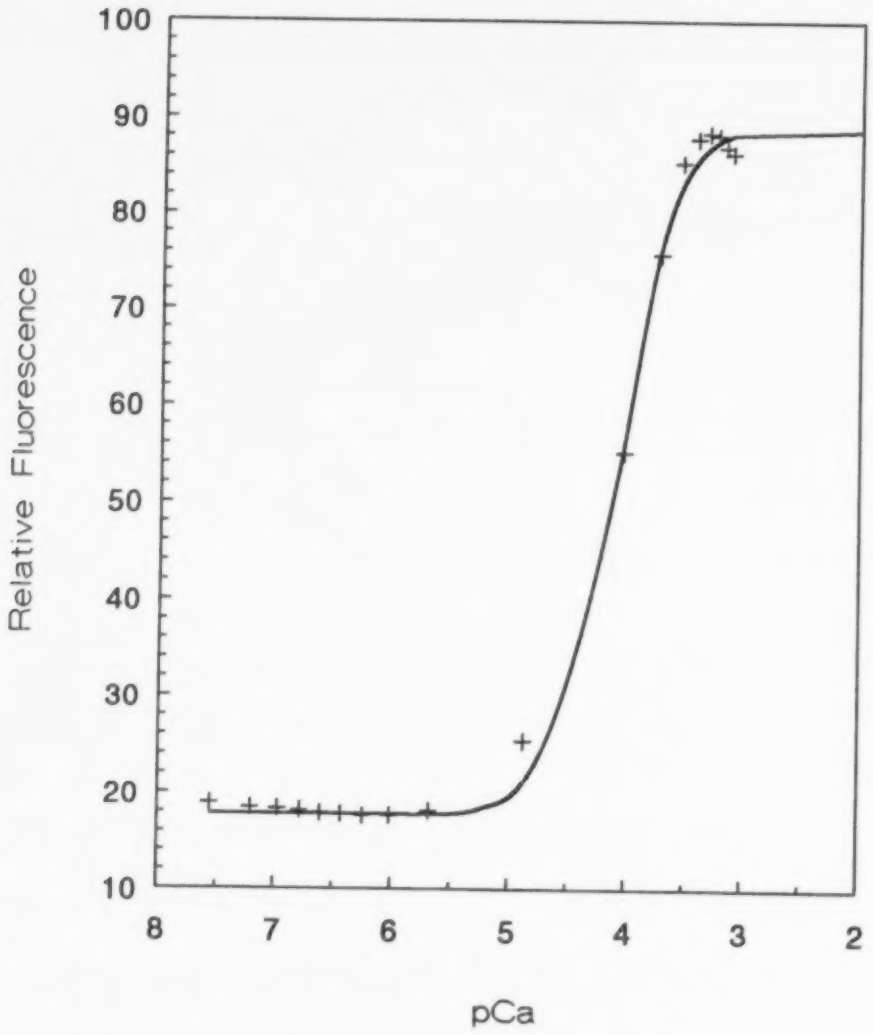

Figure 5.4: Calcium titration of the acrylodan calponin-caltropin complex, (+). The data was fit to a sigmoidal transition equation(——).

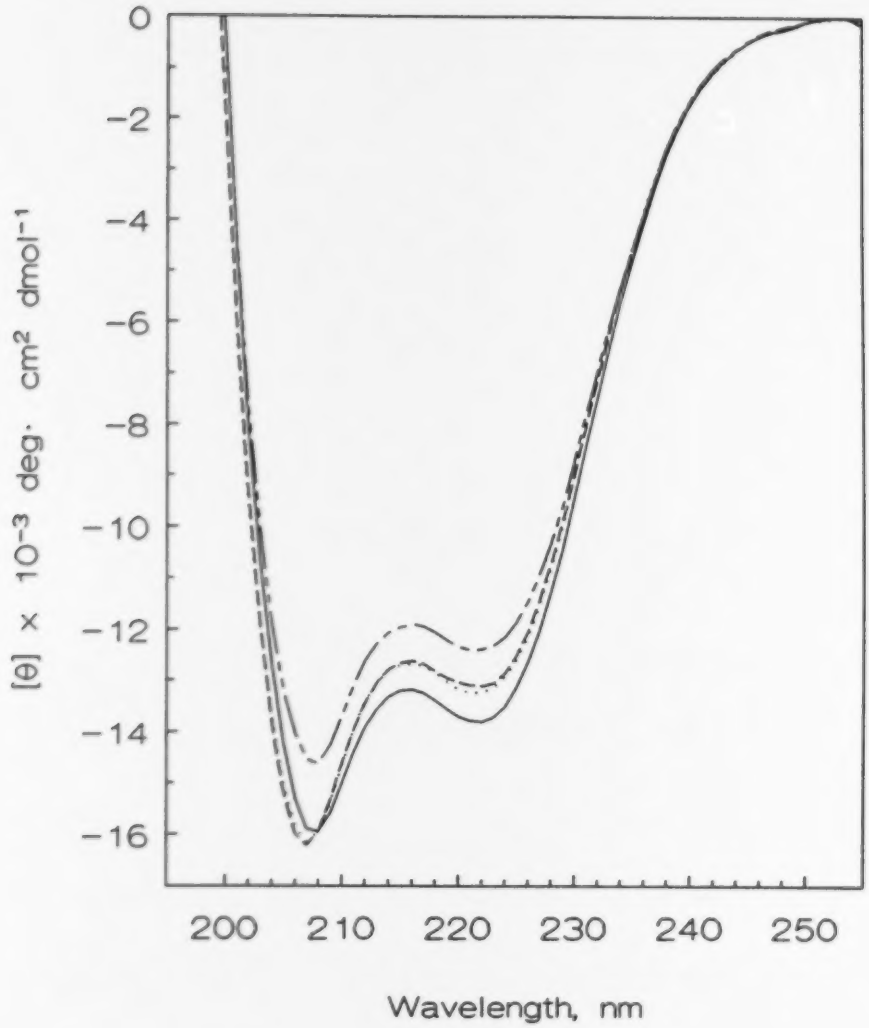

Figure 5.5: Far ultraviolet circular dichroism spectra of the observed (• • • •) and calculated (----) calponin-caltropin complex in the absence of calcium, and the observed (— — —) and calculated (——) complex in the presence of calcium.

the complex in the presence and absence of calcium were calculated in order to determine if the calculated and observed spectra are the same. A difference between the calculated and observed spectra indicates secondary structural changes occurred during complex formation. The calculated and observed spectra in the absence of calcium are superimposable signifying that no secondary structure changes occur in the absence of calcium. Analysis of these spectra by the method of Provencher and Glöckner indicate there is 43% α-helix, 25% β-sheet, 8% β-turn, and 24% remainder when calponin and caltropin are mixed in the absence of calcium. However in the presence of calcium the observed spectrum has, at 220 nm, a negative ellipticity 1590° less than that deduced for the calculated spectrum, which is much greater than experimental error (± 300°). Provencher Glöckner analysis of these experimental curves results in the calculation of 36% α-helix, 31% β-sheet, 8% β-turn, and 24% remainder. These values indicate 6% less α-helix, and 5% more β-sheet, with similar β-turn and remainder to that deduced from the calculated spectra, suggesting that there are significant structural changes that occur upon complex formation in the presence of calcium.

Guanidine Hydrochloride Denaturation.

In order to study the stability of calponin and caltropin alone relative to the complex, a guanidine hydrochloride titration was monitored by circular dichroism ellipticity measurements at 221 nm to observe the denaturation midpoint in the

presence and absence of calcium (figure 5.6). Calponin has a midpoint of denaturation of 1.25 M Gdn·HCl with a $\Delta G_D^{H_2O}$ of 3.0 kcal/mol, which is unaffected by the presence of calcium, in agreement with earlier results which showed calponin does not interact with calcium (Wills *et al.*, 1993). Caltropin in the presence of calcium has a midpoint of 2.77 M Gdn·HCl with a $\Delta G_D^{H_2O}$ of 5.45 kcal/mol. The combination of calponin and caltropin in the absence of calcium possesses a midpoint of 1.91 M Gdn·HCl and a $\Delta G_D^{H_2O}$ of 4.09 kcal/mol, and, in the presence of calcium, has a denaturation midpoint of 2.48 M Gdn·HCl with a $\Delta G_D^{H_2O}$ of 4.69 kcal/mol. These values indicate that calponin is stabilized in the presence of caltropin since the complex does not begin to denature until beyond the midpoint of calponin denaturation. The theoretical curve for the guanidine denaturation of the complex shows significant deviation from the observed denaturation profile during the early stages of the titration when calponin would be expected to denature. Later on in the titration the observed and calculated spectra are superimposable indicating caltropin stability is not affected by complex formation; however caltropin seems to confer stability upon calponin in the complex.

Temperature Denaturation of the Calponin-Caltropin Complex

The temperature denaturation profile of calponin as revealed by circular dichroism shows a sharp reversible melting transition with a T_m of 55°C (figure 5.7). Caltropin in the presence of calcium is very stable and is not completely denatured

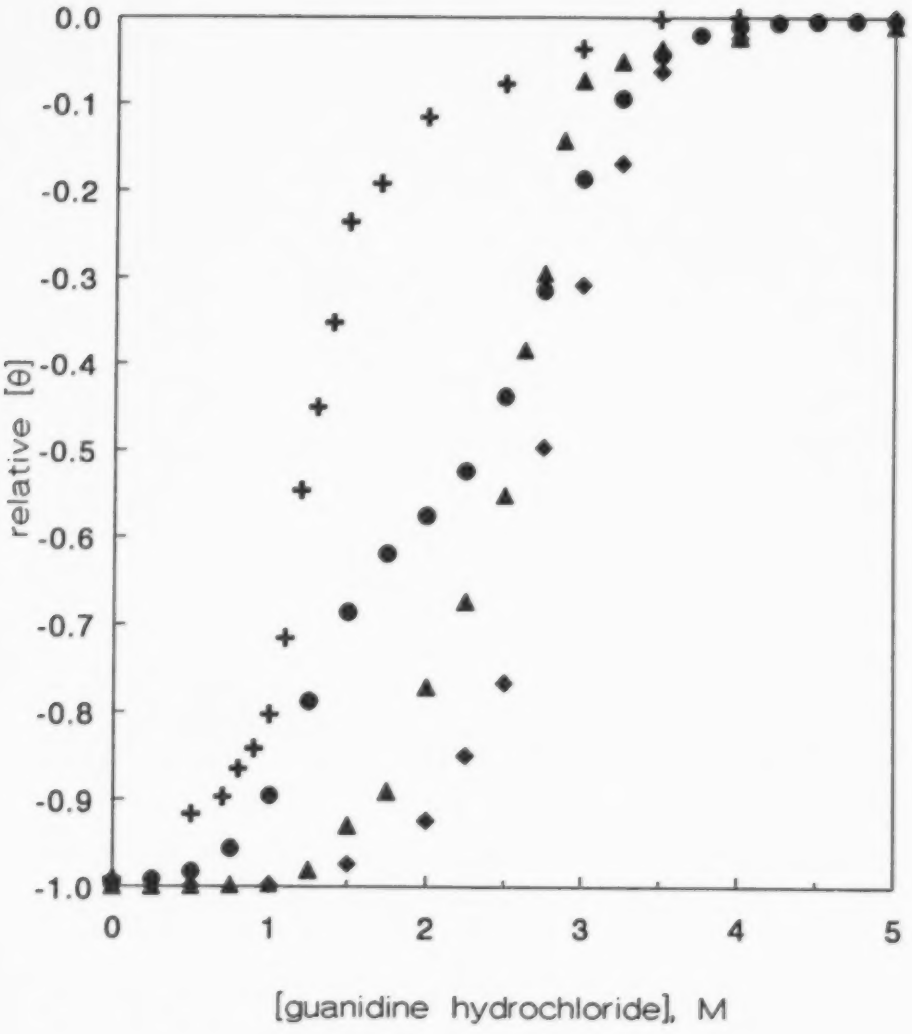

Figure 5.6: Titration of the calponin (+) , caltropin (♦) , and the calponin-caltropin complex (▲) in the presence of calcium with guanidine hydrochloride. The denaturation was monitored by circular dichroism changes at 221 nm. The theoretical denaturation profile for the complex calculated from the constituent proteins is also shown (●) . Normalized data is plotted as relative [θ] vs. concentration of guanidine hydrochloride.

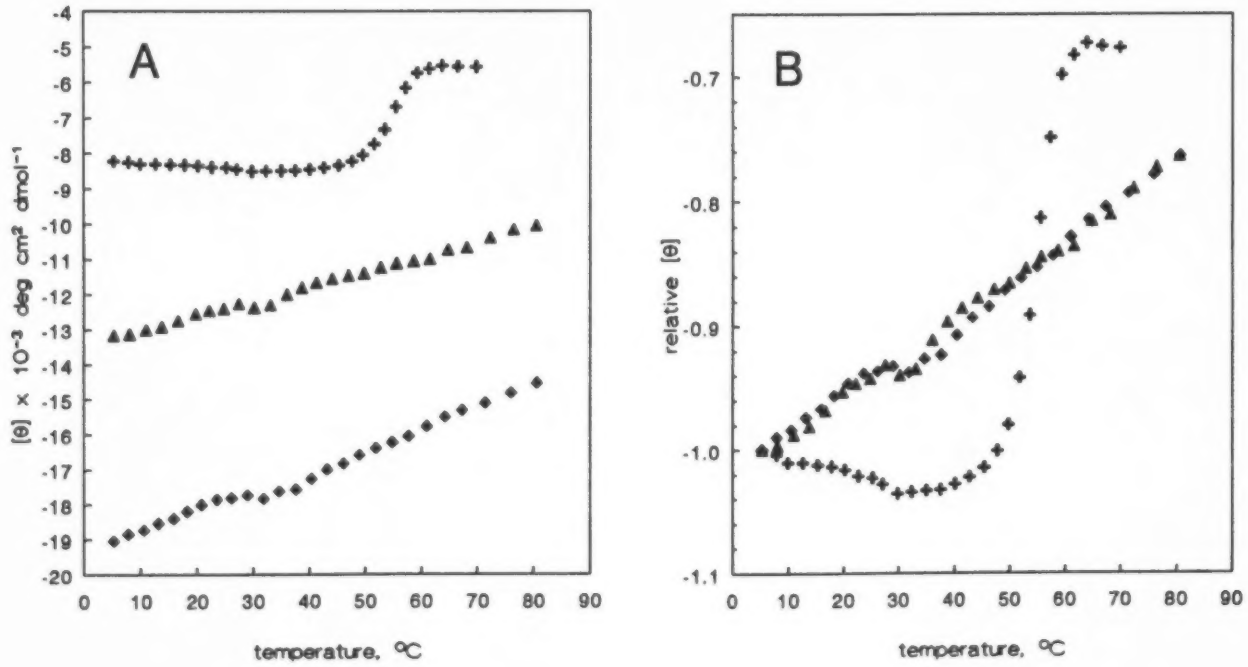

Figure 5.7: Temperature denaturation of calponin (+) , caltropin (♦) , and the calponin-caltropin complex (▲) in the presence of calcium. The denaturation was monitored by circular dichroism changes at 221 nm. Normalized data are plotted as A, absolute ellipticity, or B, relative ellipticity, vs. temperature.

even at 80°C, and does not exhibit a denaturation point at which it rapidly melts, but rather experiences a gradual loss of structure with increasing temperature. This is similar behavior to that observed for other calcium binding proteins, for example troponin C (M°Cubbin *et al.*, 1980), and calmodulin (Tsalkova & Privalov, 1980, Brzeska *et al.*, 1983). When the complex of calponin and caltropin is heated it shows very similar patterns to the denaturation of caltropin with no transition, and is not completely denatured even at 80°C. Thus, when calponin is in the complex, it does not demonstrate as great a stability at the beginning of the titration to about 40°C, but the caltropin confers stability upon calponin during the later portion of the titration such that there is no sharp melting transition at 55°C.

Effect of caltropin on ATPase Assays

In order for the calponin-caltropin complex to be an important regulatory unit, caltropin must be able to modulate calponin's inhibitory activity on the actomyosin ATPase activity. In an acto-S1 ATPase assay a 0.5 mol calponin to 1 mol actin ratio was able to inhibit ATPase 47.8%. Increasing ratios of caltropin, S-100b, and calmodulin were tested to determine to what extent these three proteins could affect calponin's activity. The ATPase assay was carried out with 0.5 mol calponin : 1 mol actin, and then increasing amounts of the relevant three calcium binding proteins were added in the presence and absence of calcium to determine if they had an effect. In the absence of calcium, calponin was able to inhibit ATPase activity with no effect of the calcium binding proteins (data not shown);

however, in the presence of calcium and calcium binding proteins there was recovery from inhibition. Figure 5.8 shows the recovery from inhibition at three ratios of calcium binding protein to calponin. The graph indicates that at all three concentrations examined, S-100b and caltropin were more effective in reversing inhibition than calmodulin. At a 2 mol ratio of caltropin to calponin, which is the ratio of these components in the complex, caltropin is able to release 63% of the inhibition, while S-100b is able to release 59% of the inhibition. Under the same conditions, calmodulin is able to release only 33% of the inhibition. Caltropin and S-100b are superior to calmodulin in releasing inhibition at all three ratios; however the greatest difference is at the lowest ratios, which would be expected to be the physiologically important ones. At 1:1 ratios caltropin releases 37% of the inhibition, S-100b 33%, and calmodulin 17%. At the higher 6:1 mol ratio, caltropin released 77% of the inhibition, S-100b 84%, and calmodulin 69%. Thus the increase in release for the 6:1 ratio is not significantly higher than the 2:1 ratio considering 3 times the calcium binding protein was used. Therefore, caltropin which is efficient at modulating calponin's inhibition, as well as being present in smooth muscle, is a prime candidate for forming a regulatory complex with calponin in a 2 mol caltropin : 1 mol calponin ratio.

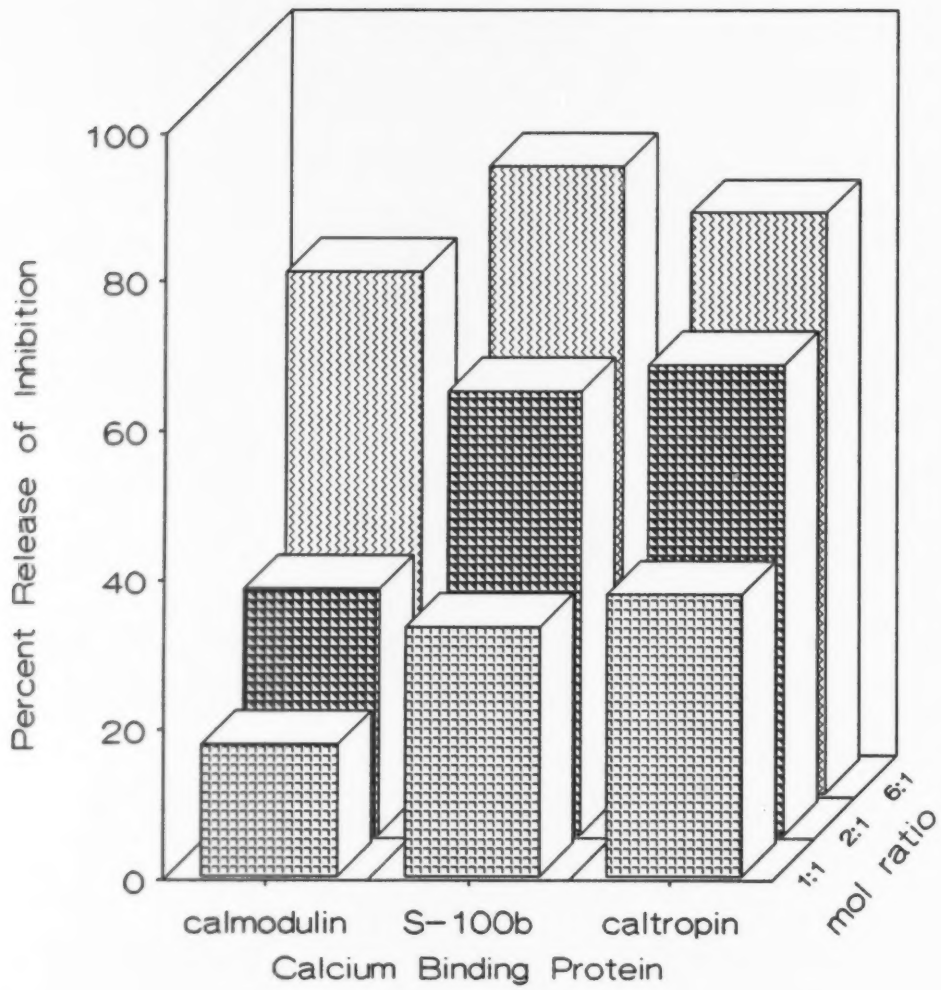

Figure 5.8: Reversal of calponin inhibition of the acto-S1 Mg^{2+}ATPase by calmodulin, S-100b, and caltropin at a 1:1 (first row), 2:1 (second row), and 6:1 (third row) mol ratio of calcium binding protein to calponin in the presence of Ca^{2+}.

SUMMARY

Currently, a great deal of work is being done to elucidate the mechanisms underlying thin filament regulation of smooth muscle contraction, and it is of interest to reconcile the relative significance of the calponin-caltropin interaction in this process. While significant progress has been made in identifying potential participants in this process, methods of regulation which explain all the data have yet to be described. Due to the similarities between calponin and caldesmon, attention has focused upon the relationship between the two proteins. It has been found that at sub-saturating concentrations relative to actin, the two proteins do not have any effect upon the function of the other, while at saturating conditions they compete for binding to actin (Makuch *et al.*, 1991, Winder *et al.*,1992). That the two proteins cannot coexist on the same filament means the two do not form a complex analogous to the troponin system, and also indicates they are not working in concert. Further exploration of both proteins is required in order to understand their respective functions.

When calponin was first isolated by Takahashi *et al.* in 1986, it was described as an actin, tropomyosin and calmodulin binding protein. A parallel to the troponin system was made and it was postulated that calponin would serve to inhibit contraction, while calmodulin would function as a calcium sensitizing agent (Abe *et al.*, 1990). Recent work revealing calponin can interact with myosin is

intriguing, however unphysiological salt conditions are required for interaction (Lin *et al.*, 1993, Szymanski & Tao, 1993). The role of calponin in binding actin has been borne out by experiments which have shown that calponin can bind to actin, inhibit actin's enhancement of myosin ATPase, and inhibit actin filament motility on immobilized myosin (Shirinsky *et al.*, 1992). Furthermore, it has been observed that calponin does not affect the phosphorylation level of myosin (Winder & Walsh, 1990), but rather seems to act by binding to actin and inhibiting myosin's interaction with actin (Abe *et al.*, 1990), thus representing a separate regulation system from myosin light chain phosphorylation. The question of the manner by which calponin's function is regulated, however, has become the subject of much controversy.

Winder & Walsh (1990) found calponin could be phosphorylated *in vitro* by both protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. Phosphorylation by either of these two protein kinases abolished calponin's inhibition of actomyosin ATPase activity. Phosphorylation also blocked the actin-calponin interaction as shown by sedimentation assays (Winder & Walsh,1990). These authors therefore proposed that calponin was regulated by reversible phosphorylation. Preliminary data on canine trachea provided evidence of calponin phosphorylation in response to stimuli in this tissue (Pohl *et al.*, 1991). However, the biological significance of this is uncertain because of a lack of confirmation of *in vivo* calponin phosphorylation by other investigators. Barany *et al.* (1991) found, using porcine carotid arterial muscles labelled with ^{32}P, that no phosphorylation of

calponin takes place in contracting or resting arterial smooth muscle, indicating that the sites of calponin available for phosphorylation *in vitro* are blocked in the intact muscle. Gimona *et al.* (1992) also observed in living smooth muscle strips from chicken gizzard and guinea pig taenia coli, labelled with $^{32}PO_4$, that no phosphate incorporation could be detected in any of the calponin isoforms whether during contraction or relaxation.

An alternative regulatory proposal to phosphorylation is regulation by calcium-calmodulin which has been shown to reverse calponin's inhibitory activity (Abe *et al.*, 1990, Makuch *et al.*, 1991). Release of calponin's ATPase inhibition by calcium-calmodulin parallels release of actin binding by calponin as shown by cosedimentation assays, indicating when calponin is bound to actin it inhibits ATPase, and when it is released it is no longer active (Abe *et al.*, 1990, Makuch *et al.*, 1991). However as much as a 10 molar excess of calmodulin over calponin is required to achieve this indicating low association constants between these two proteins. Shirinsky *et al.* (1992) found in *in vitro* motility assays that inhibition of actin filament movement over immobilized smooth muscle myosin or skeletal muscle HMM by calponin could be reversed by calmodulin but large molar excesses of calmodulin are again required to exert this reversal, which calls into question whether this is physiologically possible. Calmodulin tends to interact with proteins it regulates with affinities as high as 10^{-10} M (Klee, 1988) making this system an unlikely regulatory mechanism, and a more effective one is required. In order for a regulatory complex to be effective, it requires interaction at high

enough affinity for the formation of the complex to be biologically feasible, and it also requires sensitivity to the physiological state of the cell, so the complex can be switched on or off as the regulatory mechanism is required in the cell. Much work has been published on the calponin-calmodulin interaction. Our study suggests that caltropin, a smooth muscle calcium binding protein is capable of interacting with calponin with much higher affinity, and in so doing, regulates calponin's biological activity more effectively than calmodulin. Furthermore, since the complex only forms in the presence of calcium levels that occur in the cell during excitation, it meets the requirements of the regulatory mechanism. Thus when calponin is bound to actin, it inhibits actin activation of the myosin Mg^{2+}ATPase. Based on this study, we propose that as calcium levels increase in the cell after excitation, calcium binds to caltropin which in turn interacts with calponin. The caltropin-calponin complex no longer inhibits actin-myosin interaction and contraction ensues. Future work planned to further explore this putative regulatory mechanism includes defining the two sites of interaction of caltropin with calponin and the relevance of each of these sites.

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Chapter 6

TWO DOMAINS OF INTERACTION WITH CALCIUM BINDING PROTEINS CAN BE MAPPED USING FRAGMENTS OF CALPONIN†

†A version of this chapter has been submitted for publication.

INTRODUCTION

In order to more fully understand the interaction between calponin and calmodulin or caltropin, we have studied various fragments of calponin in order to map the sites of interaction on calponin. From the accessibility profile of calponin (figure 6.1) it is apparent there are two highly exposed regions of the molecule, of 10 amino acids or more, which may by involved in protein-protein interactions: 22-32, and 145-159. It was therefore of interest to isolate these domains to determine their binding specificity. A number of previous studies have been reported in an effort to identify sites of interaction on calponin. Winder & Walsh (1990) used NTCB, which cleaves at cysteine, to show that a 21 kDa fragment of calponin retained actin, tropomyosin, and calmodulin binding abilities in addition to the ability to inhibit the actin activated myosin ATPase. Vancompernolle *et al.* (1990) isolated the amino terminal 22 kDa chymotryptic fragment of calponin and reported it is able to bind tropomyosin. Finally, Mezgueldi *et al.* (1992) found that the carboxyl terminal 13 kDa chymotryptic fragment of calponin did not bind actin, or calmodulin, while the amino terminal 13 kDa and the amino terminal 22 kDa fragments were able to bind actin and calmodulin. On the other hand, actin would only bind to the amino terminal 22 kDa fragment which localized the actin binding region to between residues 144 and 184. When this work was carried out, assumptions on binding were made based upon one binding site on calponin for

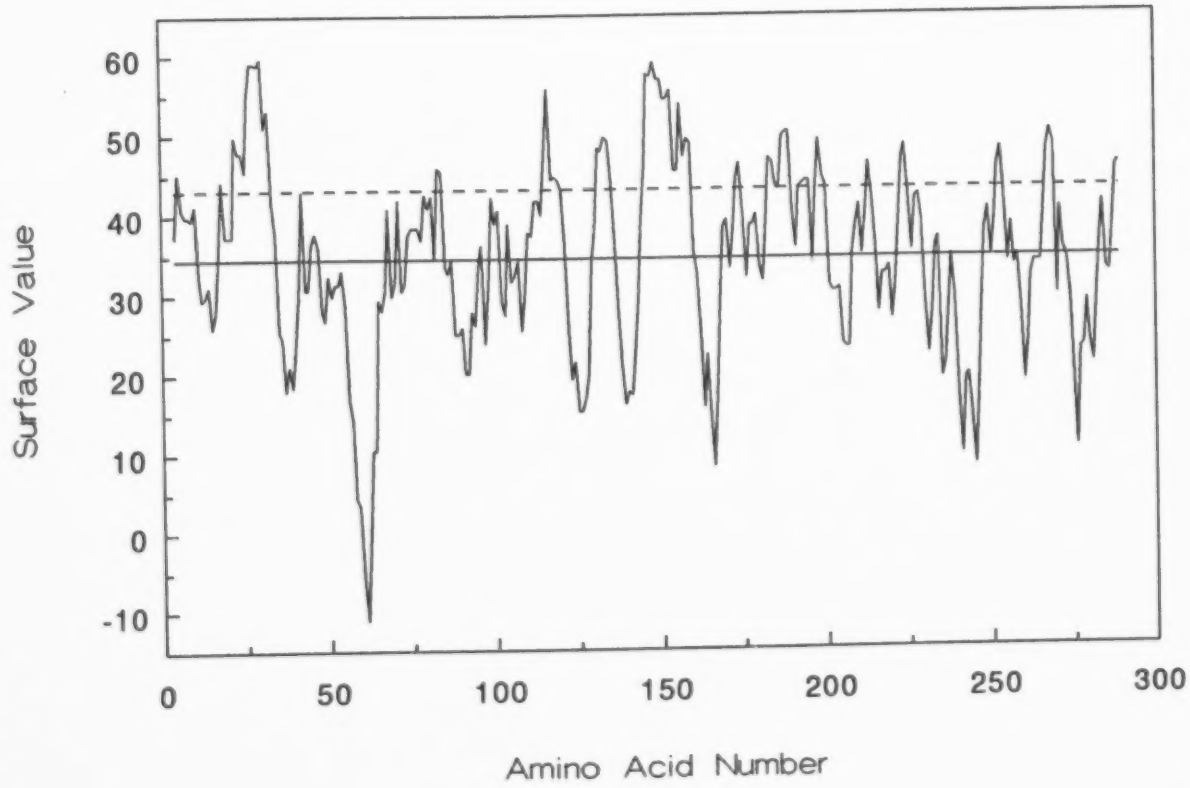

Figure 6.1: Accessibility profile of calponin calculated according to the method of Parker *et al.*(1986). Solid line represents the mean value for the entire protein, and the dashed line is mean + 25% of the difference between the mean and the maximum.

CaBP's, and it has been demonstrated that in fact two mols of calcium binding protein can bind to each mol of calponin (Wills *et al.*, 1993, 1994). Therefore it is necessary to carry out fragment studies to delineate both sites of interaction. In order to accomplish this we have studied the interaction of three fragments of calponin by their binding to caltropin and calmodulin. A C-terminal truncated calponin comprised of residues 1-228 was expressed in E.coli through recombinant techniques in order to study the effect of the loss of the C terminal portion of the molecule on stability and protein interactions. In addition residues 2-51 were isolated from the CNBr digest of calponin, and residues 45-228 were produced through recombinant techniques in order to study the complementary portion of the 1-228 mutant. We have been able to separate the two binding sites for the calcium binding proteins, assign one site to each of the smaller fragments, and characterize the interaction between these fragments and the calcium binding proteins.

RESULTS

Analytical ultracentrifugation of the C-terminal truncated calponin 1-228.

In order to determine if the region from residue 229 to the carboxyl terminus influenced the binding of calponin to the calcium binding proteins, the CP 1-228 mutant was combined with excess caltropin, and the molecular mass of the complex was determined in the analytical ultracentrifuge in the presence of

calcium. This approach has been well established in two previous papers (Wills *et al.*, 1993, 1994) as an efficient method of determining the stoichiometry of interaction of calponin in complex with a variety of calcium binding proteins. Previously we have shown that calmodulin and caltropin are able to bind to calponin in a ratio of 2 mols calcium binding protein : 1 mol calponin. It was logical, therefore, to apply this same method to determine whether CP 1-228 maintained the ability to bind two mols of caltropin. When CP 1-228 was run alone, a molecular mass of 25.7 kDa was observed (data not shown), which is within experimental error of the molecular mass calculated for CP 1-228 of 25.2 kDa, indicating that, as with the parent calponin, no aggregation of this component occurs. Previously, it had been demonstrated that caltropin does not undergo aggregation (Mani & Kay, 1990). When excess caltropin was combined with the 1-228 fragment, a molecular mass well above a 1:1 complex was observed (figure 6.2). There was not complete complex formation, as evidenced by a heterogeneous population in the centrifugation cell; molecular mass in the cell ranged from 25 kDa, which is indicative of the uncomplexed constituents, to 63 kDa, which is within experimental error of the expected mass of a 2 caltropin : 1 CP 1-228 complex of 67 kDa. Thus, truncation of the C terminal of calponin did not affect the ability of this mutant to bind 2 mols of calcium binding protein, the same stoichiometry with which the parent calponin interacts.

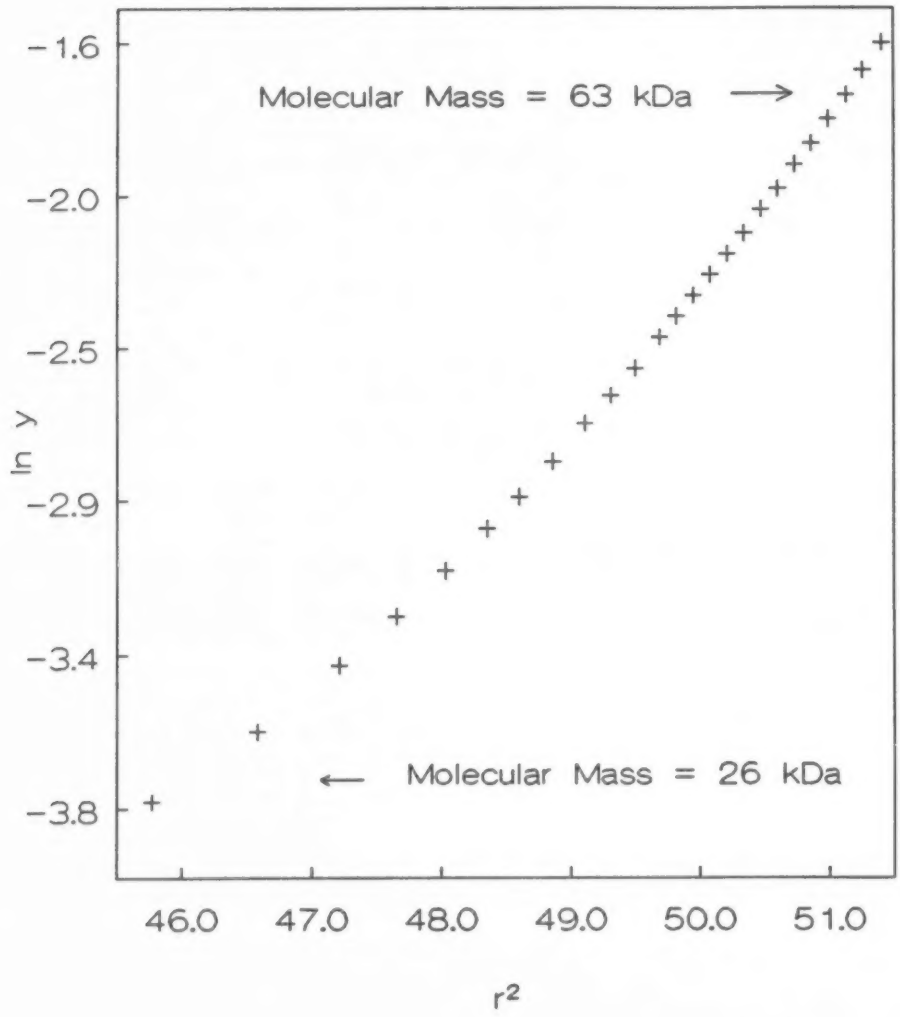

Figure 6.2: Representative plot of ln y vs r^2 for the CP 1-228 - caltropin complex. The slope of the curve is used to determine the molecular mass distribution across the cell and the molecular mass shown is the limiting mass observed at the top and bottom of the cell. The sample was combined as 2 mols caltropin : 1 mol calponin 1-228, and loaded at an initial concentration of 0.6 mg/ml. The speed was 12 000 RPM, and the buffer was comprised of 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, and 3 mM $CaCl_2$.

Denaturation of native calponin, and the fragment CP 1-228.

The temperature denaturation of CP 1-228 was monitored by circular dichroism, and the resulting spectra were compared to the temperature denaturation of native calponin. As can be seen in figure 6.3, both native calponin and the mutant 1-228 have a sharp melting transition at 55°C. CP 1-228 has reduced stability during the early stages of the titration, and loses ~10% more of the ellipticity monitored at 221 nm, indicative of a reduction in the stability of the molecule due to the C-terminal truncation. This is supported by the calculated ΔH values of 107 cal/mol for native calponin, and only 49.8 cal/mol for CP 1-228. However the similarity of the two denaturation profiles suggests the core of the molecule remains intact.

Tryptophan Fluorescence of the Calponin Fragments.

Previously, we have used an acrylodan labelled calponin in order to probe its interaction with CaBP's, since the tryptophan signal of native calponin does not respond significantly to interaction with CaBP's. The acrylodan label is attached to cys 273, the most carboxy terminal of the cysteines of calponin, and labelling the other cysteines was found to be detrimental to structural integrity (Wills *et al.*, 1993, 1994). As the fragments worked with in this study have had the carboxy terminal truncated, including cysteine 273, we examined the tryptophan fluorescence to determine if this signal would be sensitive to interaction in these

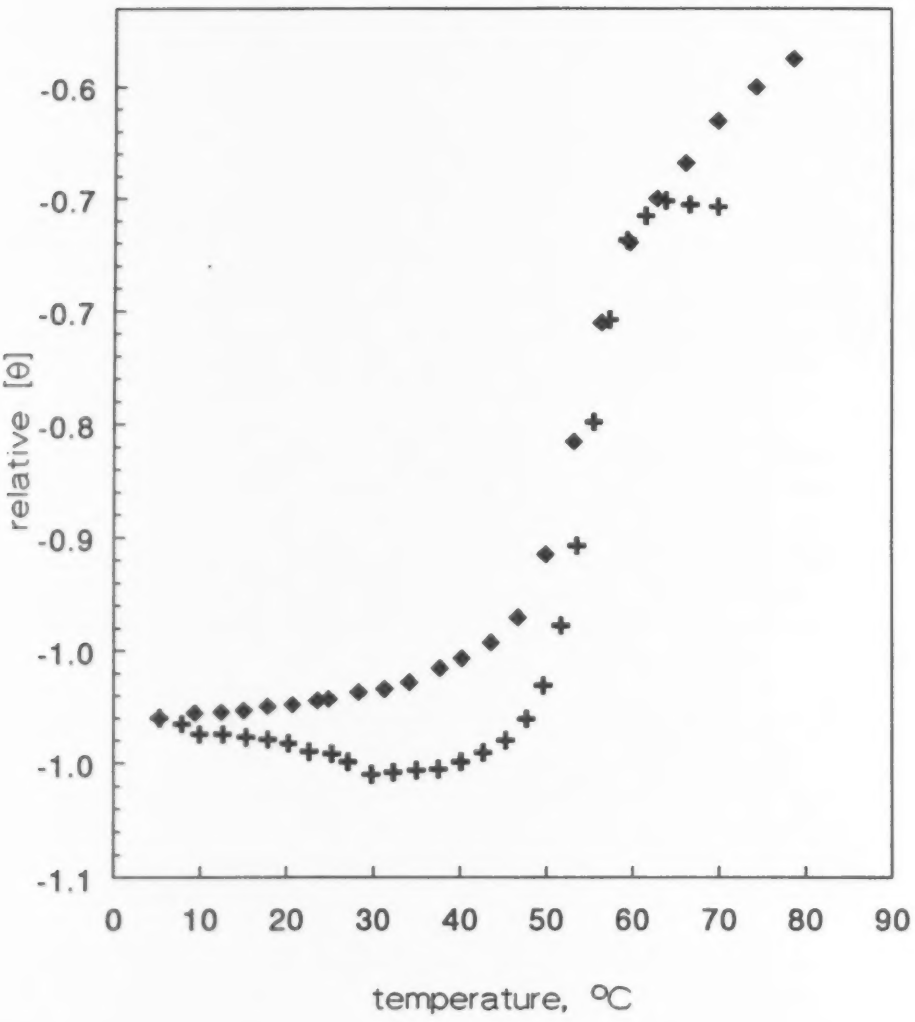

Figure 6.3: Temperature denaturation of native calponin (+), and calponin 1-228 (♦) monitored by circular dichroism at 221 nm. The concentration of protein was 1 mg/ml in 50 mM MOPS pH 7.2, 100 mM NaCl, and 1 mM DTT.

fragments. We found that in all three fragments investigated the tryptophan signal did indeed respond to interaction with calcium binding proteins.

CP 1-228 maintains both of the tryptophan residues of native calponin. Fluorescence studies on this tryptophan signal show that the peak wavelength is at 332.5 nm (figure 6.4a), similar to native calponin. Interaction with calmodulin or caltropin caused a quenching of the fluorescence. Calmodulin effected a 38 % decrease in fluorescence at the peak wavelength and blue shifted the spectrum to 325 nm, indicating an overall movement of the tryptophans to a less polar, buried environment. Caltropin decreased the fluorescence by 30% and caused a red shift in the spectrum to 335 nm indicating the tryptophans moved into a more polar, exposed environment. These fluorescence changes in CP 1-228 can be monitored as the protein is titrated with the calcium binding proteins in order to determine relative affinities of these two proteins for CP 1-228. From the titrations shown in figure 6.5a it is apparent that while calmodulin may cause a slightly greater quenching of fluorescence, caltropin still has a much higher affinity for CP 1-228, in agreement with the previously published titrations monitoring the acrylodan label on whole avian calponin (Wills *et al.*, 1994). The titration curves show a slight sigmoidal character, which we believe is due to opposing responses of the two tryptophans. From the analytical ultracentrifuge data it is known that two mols of CaBP bind to each mol of CP 1-228, and also CP 1-228 contains two tryptophans. Initially one of the tryptophans may be experiencing an increase in fluorescence, and then a lower affinity binding may be causing a decrease in fluorescence. This

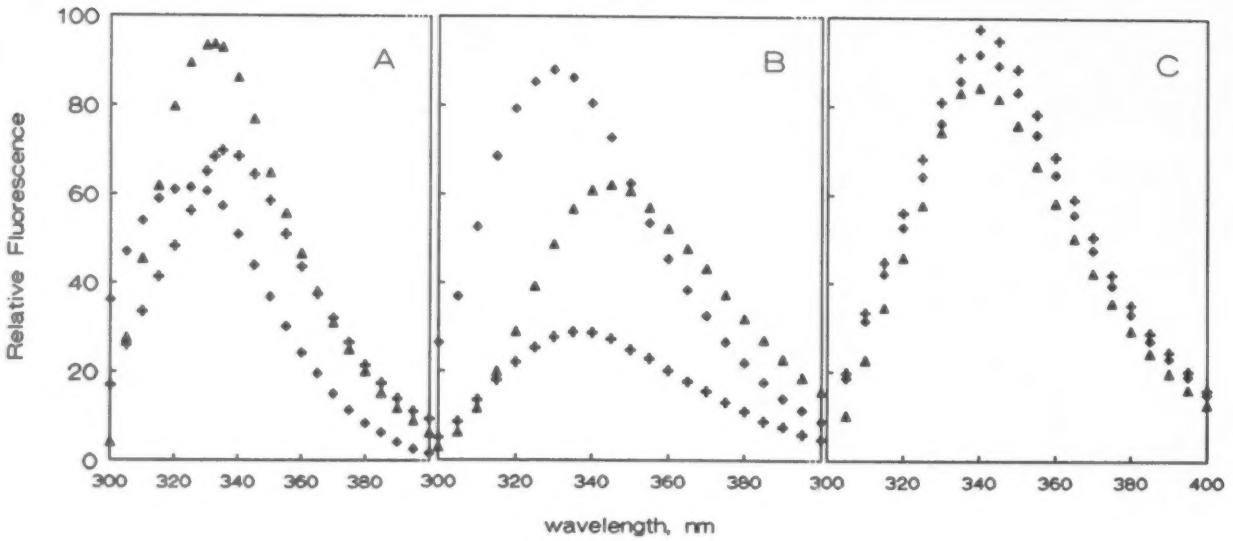

Figure 6.4: Fluorescence emission spectra of calponin fragments in the apo state or in the presence of calcium (▲), complexed with caltropin (+), and in complex with calmodulin (♦). A, CP 1-228 excited at 295 nm, B, CP 2-51 excited at 295 nm, and C, CP 45-228 excited at 300 nm. The buffer conditions were 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, ± 3 mM $CaCl_2$.

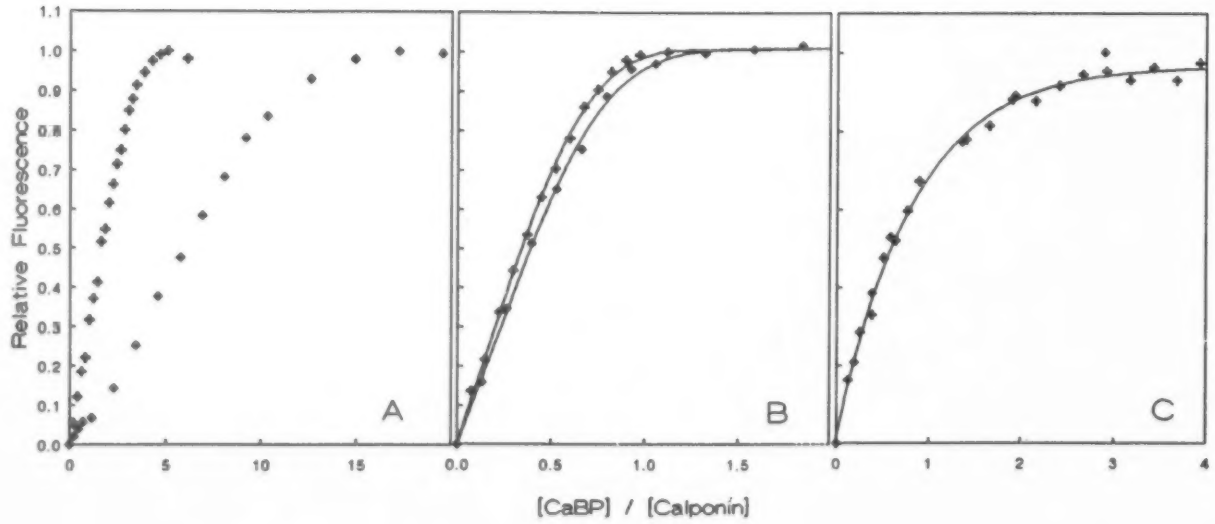

Figure 6.5: Fluorescence titration of calponin fragments with caltropin (+) and calmodulin (♦). A, CP 1-228 excited at 295 nm, and monitored at 332 nm at an initial protein concentration of 1.7 uM. B, CP 2-51 excited at 295 nm, monitored at 330 nm, at an initial protein concentration of 13.2 uM. C, CP 45-228 excited at 300 nm, monitored at 340 nm, at an initial protein concentration of 2.28 uM. The buffer conditions were 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 3 mM $CaCl_2$.

type of complicated situation, in which the contribution of the individual tryptophans cannot be quantified, precludes analysis of the data for binding constants.

The proteolytic fragment CP 2-51 possesses one tryptophan residue at position 37. The fragment was purified by binding in a calcium specific manner to a calmodulin affinity column demonstrating it was capable of binding to calcium binding proteins. By monitoring the fluorescence of this tryptophan, the binding of CP 2-51 to both calmodulin and caltropin was confirmed. CP 2-51 has a peak wavelength of emission at 342 nm in the presence and absence of calcium which indicates the tryptophan is in a highly exposed environment (figure 6.4b). When calmodulin is added to CP 2-51 there is a 50% increase in the fluorescence emission at the peak wavelength, together with a shift in the maximum to 330 nm. Caltropin also causes a blue shift in the spectrum to 332 nm; however, rather than an increase in fluorescence intensity, caltropin quenches the fluorescence by 50%. Thus, in this case, there is a difference both in the quantitative response and the qualitative effect of these two proteins upon this fragment of calponin. These changes can be titrated by monitoring the tryptophan fluorescence as increasing amounts of the calcium binding proteins are added. Figure 6.5b shows the results of this titration, normalized so that binding affinities can be compared directly. From the titration it is evident that 2 mols of CP 2-51 are able to interact with each mol of calcium binding protein since 60 % of the titration is over by the time a 0.5:1 ratio of CaBP to CP 2-51 is added. However there is only a single class of binding site apparent as the curve is not biphasic. This in not surprising since this peptide

has a molecular mass of only 5300 Da while calmodulin has two hydrophobic patches, one on each domain, each of which can bind to certain drugs with a hydrophobic ring structure (reviewed in Means *et al.*, 1991). Caltropin is a dimer, resulting in one peptide interacting with each component monomer. Binding affinities for such curves can be calculated by assuming that when 50 % of the fluorescence change has occurred, 50 % of the protein is bound, and then calculating the free CaBP at this point. Application of this method results in a Kd of 1.2 uM for caltropin, while 1.8 uM is found for calmodulin, indicating that this fragment is barely able to differentiate between calmodulin and caltropin by binding to one tighter than the other, unlike the parent calponin and the fragment CP 1-228 which show much greater affinity for caltropin over calmodulin (Wills *et al.*, 1993,1994).

The tryptophan fluorescence of the fragment 45-228 can be used to demonstrate that this portion of calponin is also able to bind to CaBP's. This fragment also possesses a single tryptophan at position 82 which has a peak of fluorescence at 340 nm indicating this fluorophore is in an exposed polar environment. The tryptophan responds to the interaction with calmodulin and caltropin by increasing its intensity with no concommitant wavelength shift. Caltropin is able to increase the fluorescence of the fragment by 18%, while calmodulin is able to cause a 9% change (figure 6.4c). These are not the same qualitative effects observed with CP 2-51, supporting the hypothesis that the tryptophans of CP 1-228 are not experiencing the same environmental effects.

When these changes are titrated with the calcium binding proteins (figure 6.5c), and the results normalized, it is apparent that the binding affinity of CP 45-228 for these two CaBP's is very similar, and using the method referred to above to calculate binding constants, and assuming 1 mol per mol interacts, a Kd of 0.07 μM can be calculated. It is apparent that the differential affinity the parent displays for the various CaBP's is not inherent in these smaller fragments, and further work must be done on the parent, with the full complement of possible interactions intact in order to further investigate this behavior.

Secondary Structure Studies on Calponin and its Fragments.

Circular dichroism was performed on the fragments of calponin in order to compare their secondary structure with that of the intact calponin molecule. In addition the CD spectra of the complexes of these fragments with caltropin were determined in the presence and absence of calcium, and compared to the theoretical spectra, generated by summing up the constituent spectra, in order to determine the type of structural changes that occur upon complexation. In figure 6.6a the CD spectra of native calponin, calponin 1-228, the complex of calponin 1-228 with 2 mols of caltropin in the presence of calcium, and the theoretical complex of 1-228 with caltropin in the presence of calcium are presented. The CD spectrum of chicken gizzard calponin has been published previously (Wills *et al.*, 1993), and here we present the spectrum of porcine calponin to demonstrate the CD spectrum of smooth muscle calponin does not significantly vary from species

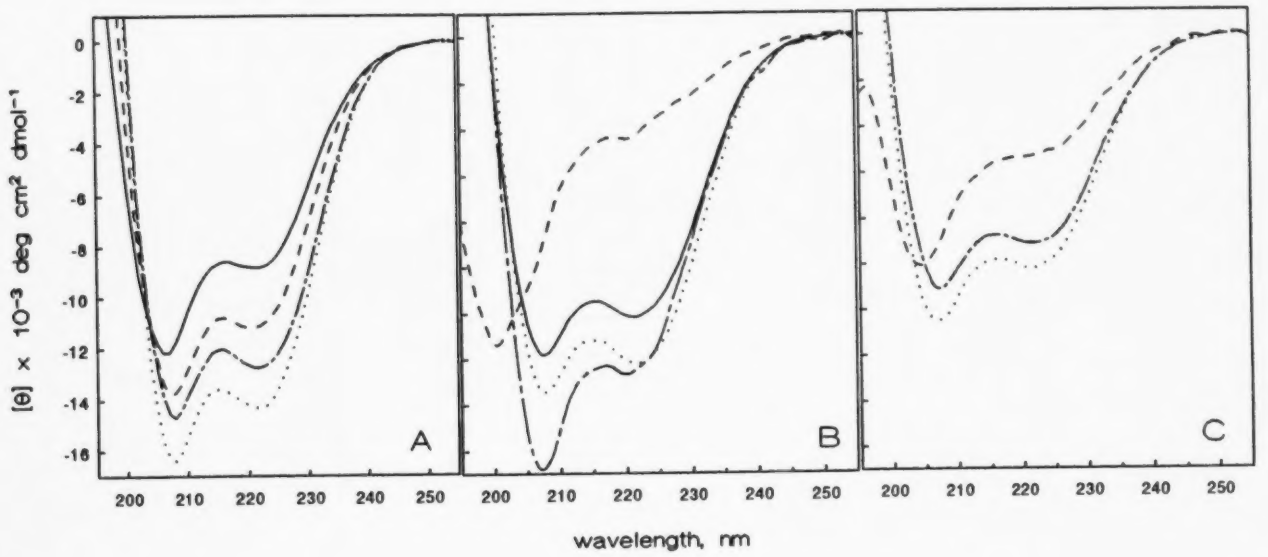

Figure 6.6: Far ultraviolet circular dichroism spectra of calponin fragments. A, spectrum of CP 1-228 (----), native porcine calponin (——), CP 1-228 in complex with caltropin (— —), and the theoretical complex of CP 1-228 with caltropin (• • • •). B, CP 2-51 (----), 2-51 with 50 % TFE (——), in complex with caltropin (— —), and the theoretical complex with caltropin (• • • •). C, CP 45-228 (----), in complex with caltropin (— —), and the theoretical complex with caltropin (• • • •). The buffer conditions employed were 50 mM MOPS pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 3 mM $CaCl_2$ was present for the complexes.

to species. The ellipticity at 221 nm varies by 328° between these species which is within experimental error (+/- 300°) and does not represent significant structural differences. The mutant mouse calponin fragments do not vary significantly in sequence from the porcine used as control; there are only two amino acid differences in the 1-228 fragment, an arginine to a glutamine and an arginine to a lysine (Strasser *et al.*, 1993). Provencher Glöckner analysis of the CD spectra was performed in order to evaluate the secondary structure content of the proteins and the complexes. The results are tabulated in table 6.1. The results of the analysis of the 1-228 species indicate this fragment still has a high level of structure despite the C-terminal truncation. Since we had established 2 mols of CaBP bind to each mol of CP 1-228, we used this ratio of proteins in order to establish the CD spectrum of the complex and compared it to the theoretical one in order to determine the type of secondary structure that changes upon complex formation. In the absence of calcium the observed and theoretical spectra were superimposable indicating no secondary structural changes occurred (data not shown). In the presence of calcium however, there was a 1580° smaller ellipticity in the observed complex vs. the theoretical spectrum at 221 nm, indicating 6% less α-helix, and 11% more β-sheet in the observed versus the calculated complex.

Figure 6.6b shows the CD spectra of the CNBr proteolytic fragment of CP 2-51. This fragment has low α-helix, high β-sheet, β-turn, and remainder. In order to determine if more helix could be induced in this fragment under the appropriate conditions, we added 50% TFE, a known helix inducer (Jasonoff & Fersht, 1994).

Table 6.1: Secondary structure predictions.[a]

Protein	α-helix	β-sheet	β-turn	remainder
porcine CP	33	23	16	27
CP 1-228	42	15	11	31
CP 1-228 + CT	43	26	10	21
theoretical	49	15	08	28
CP 2-51	13	43	22	22
CP 2-51 + 50% TFE	48	27	03	22
CP 2-51 + CT	47	22	06	25
theoretical	38	21	10	31
CP 45-228	17	29	26	28
CP 45-228 + CT	30	35	18	17
theoretical	35	25	11	29

[a]Analysis of the circular dichroism spectra are expressed as percent of total structure. The complexes are in the presence of Ca^{2+} as described with the individual experimental results, and the theoretical is the predicted amounts based on summing up the constituent proteins assuming no secondary structural changes occurred upon complexation.

The apparent helical content of CP 2-51 increased significantly to 48%, indicating that this fragment is certainly capable of taking on helical structure in the native protein, or upon interaction with another protein. The accessibility profile (figure 6.1) of calponin indicates this region does not contain any significant non-accessible regions, so it is not surprising that it is not fully structured when free in solution. When CP 2-51 was combined with caltropin in the absence of calcium, the CD spectrum was superimposable with the theoretical spectrum indicating no secondary structural changes occurred (data not shown). In the presence of calcium however, there was a 1990° greater ellipticity at 221 nm than predicted, most likely due to the induction of helix in CP 2-51 upon interaction with caltropin. Provencher Glöckner analysis indicated an increase of 9% helix over the predicted structure.

Figure 6.6c contains the CD spectra of the recombinant CP 45-228. The accessibility plot (figure 6.1) indicates there are highly inaccessible residues in this region indicating it may form part of the hydrophobic core of the protein. When combined with caltropin in the absence of calcium, there was no change in secondary structure compared to the theoretical spectrum generated for this complex (data not shown). However, in the presence of calcium there is a 1090° smaller ellipticity at 221 nm than predicted , indicating structural changes occurred upon interaction. The Provencher Glöckner analysis indicates a 5% decrease in α-helix, and a 10% increase in β-sheet. These changes are very similar to those observed in the complex of native calponin with caltropin (Wills *et al.*, 1994).

DISCUSSION

Progress is continuing on elucidating the details of calponin's biological role, especially with relation to its inhibition of the actomyosin interaction. Horiuchi and Chacko (1991) found that calponin causes a decrease in the V_{max} of the actomyosin ATPase, without a substantial decrease in the K_{ATPase}. This finding suggests calponin is causing a structural change in actin which is affecting the catalytic state of the reaction, rather than sterically blocking the interaction of myosin and actin. A similar conclusion is indicated by studies that covalently crosslinked actin to S1, wherein calponin was still able to inhibit the ATPase (Miki *et al.*, 1992) The work of Shirinsky *et al.*, (1992) has shown, by monitoring actin filament motility over immobilized myosin, that calponin inhibited filament movement in an all or none fashion, rather than producing a graded inhibition of filament velocity, and this finding has been supported by the recent study of Haeberle (1994) in which calponin was found to inhibit filament velocity, while at the same time increasing the force of the crossbridges, and strengthening the actin-myosin binding. Haeberle proposed that this indicates calponin is enhancing the strong binding state of actin and myosin leading to a load bearing complex, and could therefore contribute to the latch state of smooth muscle. Regulation of calponin's function is not well understood, and proposals such as phosphorylation of calponin as a mechanism of regulation are highly controversial. In this study, the interaction

of calponin with the calcium binding protein caltropin has been probed. This interaction is of great potential interest in smooth muscle regulation as caltropin has been demonstrated to be capable of regulating calponin's activity in *in vitro* ATPase assays (Wills *et al.*, 1994)

The present study has demonstrated the utility of calponin fragments in studying the interaction of calcium binding proteins with calponin in terms of the characterization of the interactions along with identifying the domains on calponin where the interactions occur. Native calponin tryptophan fluorescence does not respond significantly to the interaction of calponin with calcium binding proteins; however, these fragments of calponin do respond with a change in the tryptophan fluorescence which signifies that for the first time intrinsic fluorescence can be used to monitor the interactions allowing investigation of complex formation in the absence of probes modifying the protein. Thus, while the deletion of residues 229 to the carboxyl terminal does not affect the ability of calponin to inhibit ATPase as shown with studies of other fragments [Winder & Walsh (1990b), Mezgueldi *et al.* (1992)], or to bind to CaBP's as shown in this study, the carboxyl terminal fragment certainly interacts with other portions of the molecule in order to alter the tryptophan fluorescence response. This is also indicated by acrylodan labelled cysteine 273 of calponin, which is located in this truncated carboxyl terminal region, being extremely sensitive to interaction with CaBP's despite this region not being required for interaction. The fluorescence of CP 1-228 indicated a different type of response with calmodulin which caused a blue shift suggesting a more

protected environment, as compared to caltropin which caused a red shift, suggesting movement of the tryptophan into a more solvent exposed, polar position. This differential response was not observed with the acrylodan labelled calponin in which all of the calcium binding proteins caused a blue shift of the fluorescence due to acrylodan on cysteine 273 signifying its movement into a less exposed environment. This difference in the effect upon the tryptophans between these two calcium binding proteins may be due to the dimeric nature of caltropin as opposed to the monomeric nature of calmodulin, and suggests the tryptophans may be used in the future as a sensitive probe in studies dedicated to why caltropin binds with higher affinity to calponin than calmodulin does and the type of structural changes that binding induces in calponin. The complicated tryptophan fluorescence response of CP 1-228 in which the two tryptophans may be experiencing opposing environments, together with the lack of tryptophan response of native calponin may be further investigated by the replacement of the tryptophans with probes of altered fluorescent properties such as 5-hydroxytryptophan (Hogue *et al.*, 1992), or the non fluorescent analogue 4-fluorotryptophan (Bronskill & Wong, 1988). This would allow the response of the individual tryptophans to be examined, each one of which appears to be located in a domain of interaction with the CaBP's, and may well produce very informative results about the relative behavior of these two binding events.

Through the use of chemical cleavage of calponin, and recombinant techniques two smaller fragments of CP 1-228 were made, CP 2-51 and CP 45-

228, each of which is able to bind to calcium binding proteins. Thus the two domains of interaction between calponin and the CaBP's have been isolated. The fluorescence titration of CP 2-51 with caltropin and calmodulin cannot be fit to a 1:1 binding, and it is evident 2 mols of this peptide are able to bind to each mol of calcium binding protein. While this ratio of interaction would not be expected based on the stoichiometry of interaction of the native parent proteins, it is not an unreasonable result considering caltropin is a dimer and may well possess two equivalent sites of interaction, and calmodulin has a hydrophobic patch on both its N-terminal and C-terminal domains (Strynadka & James, 1989). Since the peptide is relatively small, with a molecular mass of 5300 Da, one would not expect the same steric hinderance to prevent both sites of interaction that one would expect to find in the parent molecule of 32 kDa. The phenomenon of two interaction sites has been observed before. For example, the skeletal muscle protein troponin I has been shown to bind to either the N or C terminal domain of both troponin C and calmodulin (Swenson & Fredricksen, 1992, Lan *et al.*, 1989). In addition, the peptide melittin has also been shown to bind to both the N and C terminal domains of calmodulin (Seeholzer *et al.*, 1986), and the NMR structure of calmodulin in complex with a peptide of MLCK shows contact with both the amino and carboxyl domains of calmodulin (Ikura *et al.*, 1992). Most recently, an analogous situation was demonstrated by Marston *et al.* (1994), in which two mols of a caldesmon peptide are able to interact with one mol of calmodulin, while this is not the stoichiometry achieved with the intact proteins. Interestingly, it has been

demonstrated in some cases, that a helix is the motif used for interaction with these calcium binding proteins, as in the case for melittin mentioned above, and we have shown in this study that CP 2-51 is capable of having helix induced in it under the appropriate conditions. Further, the complex of CP 2-51 with caltropin exhibits much greater helix than would be predicted suggesting the peptide may indeed be taking on a helical conformation upon interaction.

The fluorescence titrations of CP 2-51 and CP 45-228 with the calcium binding proteins did not show the large preference for interaction with caltropin over calmodulin that the native protein is capable of demonstrating (Wills *et al.*, 1993, 1994). However, the mutant CP 1-228 was still able to exhibit this preference. This indicates that contributions from the various domains of the protein is an important determinant in these interactions, and a number of interactions from various parts of the molecule play a role. Thus the information obtained in this study must be used to selectively modify various residues within the intact calponin molecule in order to delineate which site is the high affinity site, which site is the low affinity site, and the biological relevance of each of them.

The secondary structure analysis of the fragments of calponin indicate that the secondary structural content changes upon interaction with caltropin, which provides further evidence of complex formation. This study has looked at the N terminal 228 amino acids. In the future it will be of interest to pursue studies on the C-terminal regions of calponin the function of which remains to be resolved. Secondary structure prediction algorithms indicate this region contains β-sheet and

turns with little or no α-helix (Strasser *et al.*, 1993). In future studies involving the interactions and regulation of smooth muscle, these changes in structure should help to characterize the behavior of the calponin molecule when it inhibits actin activation of ATPase compared to when it is regulated.

As an increasing number of calponin isoforms are being identified, their sequences are being compared in order to determine the biological significance of various regions of the molecule (Vancompernolle *et al.*, 1990, Takahashi & Nadal-Ginard, 1991, Strasser *et al.*, 1993, Nishida *et al.*, 1993, Applegate *et al.*, 1994). It therefore becomes increasingly important to understand the domains of calponin and their sites of interaction with various proteins so that sequence variations can be related to function. In order to get an accurate picture of where these interactions are occurring it is important to incorporate the new information presented in this study demonstrating two sites of interaction with CaBP's. Other work in which mapping of binding sites for actin, tropomyosin, and calmodulin have been performed produced a map of binding sites (Mezgueldi *et al.*, 1992). Figure 6.7a shows our revised map based upon all the information to date. We have demonstrated that CP 1-228 which has the carboxy terminal truncated is still able to bind 2 mols of CaBP, indicating that the loss of 64 amino acids does not affect this function. Furthermore we have demonstrated that this truncation does not greatly affect the stability of calponin indicating it may not contribute to the core of the calponin molecule, however the tryptophan fluorescence results indicate the carboxyl terminal portion certainly has an effect upon the functional portion of

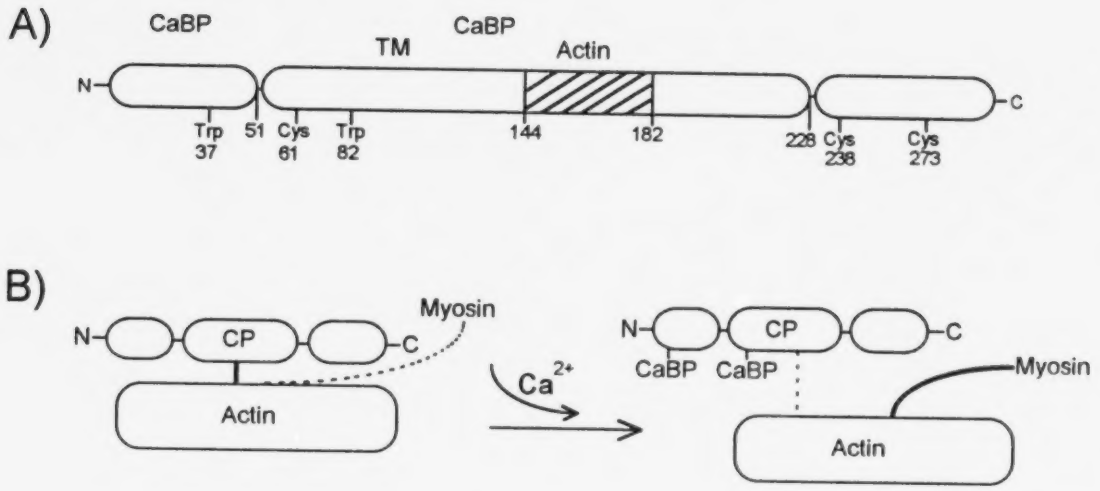

Figure 6.7: A, map of the domains of calponin as determined in this study using fragments of calponin, and the putative sites of interaction with actin, tropomyosin, and the calcium binding proteins. B, proposed model for regulation of the actin-calponin interaction by the calcium binding proteins.

calponin. Winder & Walsh (1990) found that a 21 kDa cleavage product of NTCB was able to bind to actin, calmodulin, and tropomyosin, and this fragment would presumably represent residues 61-238. It has also previously been shown that a 22 kDa calponin fragment, from residues 1-184, maintains all of the binding interactions of calponin, and that the 13 kDa C-terminal portion of calponin did not interact with tropomyosin, actin or calmodulin (Vancompernolle *et al.*, 1990, Mezgueldi *et al.*, 1992). Our results are in line with these studies wherein we find that loss of the C terminal does not cause loss of interaction with CaBP's. Previously the information has been interpreted to mean that the CaBP interaction site can be localized to amino acids 61-144. We postulate that this is not so because that interpretation was based on one CaBP site of interaction. We show in this work that there is one site of interaction between residue 2-51, which would be observed in the studies on the 1-144 fragment, and the 1-184 fragment used in the work of Mezgueldi *et al.*(1992). Furthermore, the fact that 1-144 bound to calmodulin does not preclude the presence of a second binding site in the 144-184 region. The observation of Mezgueldi *et al.* (1992), that calmodulin bound to the crosslinked actin and calponin 52-168 complex, would suggest 52-168 contains the second binding site we are detecting with our 45-228 fragment, and which Winder & Walsh observed with their 21 kDa fragment. This second CaBP interaction site may well be close to or even overlap the actin binding region of 144-184. The observation of Winder & Walsh (1990) of a 9 kDa fragment which did not bind to actin, tropomyosin, or Ca^{2+}/calmodulin must presumably have been generated

from the residues C-terminal of cysteine 238, and would not be contained within the fragments used in our study.

Figure 6.7b shows a model of how these proteins might interact. We propose that the binding site for caltropin on fragment 45-228 may overlap or interfere with the binding site for actin demonstrated to be in this region, and as such is responsible for the ability of caltropin to interfere with calponin's ability to inhibit the actin activated myosin ATPase. The observation of an additional site of interaction in the amino terminal of calponin allows for the exploration of its function to be addressed. We suggest the second site of interaction on fragment 2-51 may serve to modulate either: (i) calponin's binding to actin, (ii) a post-translational regulatory event as suggested by Naka *et al.* (1990), who showed that phosphorylation of calponin was inhibited by the presence of calcium/calmodulin, or (iii) another interaction in which calponin is involved and yet to be determined. If, as has been suggested, calponin is involved in cytoskeletal structure regulation (Takeuchi *et al.*, 1991, North *et al.*, 1994), caltropin binding to either of these two sites may well regulate or modulate interaction with other cytoskeletal proteins. This study lays the groundwork for future work which will involve further delineation of both sites of interaction and their respective functions. Determination of the functional abilities of calponin with specific sites, such as the two calcium binding protein interaction sites, appropriately modified, will aid in resolving the questions of calponin's function and its role in smooth muscle contraction.

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Chapter 7

GENERAL DISCUSSION

Initially the aim of this project was to characterize the interaction of calponin with calmodulin, as well as investigate the interplay of this interaction upon other calponin-protein interactions, and establish the role this played in the thin filament linked regulation of smooth muscle. However, the calponin-calmodulin interaction proved to be more complicated than originally anticipated, and the project evolved into defining the ability of a variety of calcium binding proteins to interact with calponin, establishing the nature of the interaction, and finally, through collaboration, the opportunity to use mutants, in addition to fragments produced in our own lab, to begin mapping the calcium binding protein sites on calponin. When the project was initiated there were perhaps 4 or 5 papers published on calponin, whereas the field is now beginning to open up, and some very interesting results are being documented. The result of this emergence of new information in a relatively novel field, is that ideas concerning the role of calponin in smooth muscle, its relationship to caldesmon, and indeed, the entire question of how latch bridge and sustained tonic contraction of smooth muscle is achieved is continually evolving, and the available information must constantly be reevaluated in light of new studies. One of the directions this evaluation is taking is that the original ideas concerning calponin's role in the cell are being rethought. Fortunately, the scope of the project undertaken, was a general characterization of the phenomena of the interaction, and the information obtained should be useful in whatever context calponin function turns to in the future. There are still many questions to be

answered concerning the exact protein which utilizes the calcium binding sites characterized in this study, and the role of this interaction in calponin's behavior. The results presented in this thesis have laid some of the groundwork towards a more detailed understanding of calponin's function. As more knowledge is acquired about the complexes calponin forms, studies will progress to the level achieved in skeletal or cardiac muscle, where specific domains of the proteins involved in regulation can begin to be assigned such roles as affecting cooperativity or calcium affinity. In addition modifications of the protein such as phosphorylation can be investigated for their role in acceleration of contraction or as a memory for those fibres recently contracted (Sweeney *et al.*, 1993), such that many of the confusing results being documented about calponin will start to be understood. More parallels will be drawn between skeletal and smooth muscle, where originally it was thought that skeletal and cardiac muscle were controlled at the level of the thin filament, while smooth muscle was believed to be solely regulated at the level of the thick filament by myosin phosphorylation. As time progresses, studies emerge giving smooth muscle thin filaments a role in smooth muscle contraction, and phosphorylation of the myosin light chains in skeletal and cardiac muscle is beginning to be assigned the role of potentiating forces, and increasing efficiency of muscle contraction during prolonged contraction cycles (Sweeney *et al.*, 1993). The regulation of the various muscle types seems to be merging into variations of the same theme which is merely adapted to meet the needs of the individual muscle types rather than dramatically altered. This

adaptation will extend beyond defining smooth, skeletal, and cardiac muscle types, and as in skeletal muscle where slow and fast twitch types exist, various smooth muscles will be assigned unique regulatory behaviours, for instance, in the situation with the intestinal tract where constant cycles of contraction occur, versus the respiratory tract in which the muscle functions to maintain tone for long periods of time.

As details regarding the properties of calponin and caldesmon emerge, the remarkable number of similarities continues to grow. Most recently results concerning the interaction sites of calmodulin upon caldesmon were published by Marston *et al.* (1994). These investigators localized two calcium/calmodulin binding sites on caldesmon, both in the carboxyl terminal end which contains many of the putative phosphorylation sites. One is near the actin binding site in the fragment which inhibits ATPase, and which calcium/calmodulin can reverse the ATPase inhibition. The second binding site is localized further towards the amino terminus and has unknown function. This is very similar to the calponin situation in which two CaBP's can interact, and one is in the same domain as the actin binding site and regulatory region, while the other is in the amino terminus region. Assignment of which of these sites is responsible for the ability to reverse calponin's inhibition has not been achieved; however one may speculate it would probably be the interaction site near the actin binding site which would be responsible. The second binding site in both calponin and caldesmon may arise for similar reasons, ie. a modulatory role, regulating a modification such as

phosphorylation, or be a non-specific site as a result of the protein having a common binding motif of which the primary function is interacting with another protein. It has been shown in another system, that of the interaction of microtubule associated protein tau with microtubules, that the interaction can be modified by phosphorylation, a process which also modulates calcium/calmodulin binding to tau (Ksiezak-Reding *et al.*, 1994). Thus it is possible that the role of calcium binding proteins interacting with calponin may not provide a direct regulatory function, but may modulate other regulatory functions. It will be of interest in the future to determine if in calponin the myosin interaction site is in the amino terminus region near the second calmodulin binding site. Marston *et al.* (1994) also demonstrate that the two binding fragments of caldesmon do not compete with each other on the calmodulin molecule indicating two sites of interaction on calmodulin. This is in line with our results which indicate the amino terminal peptide 2-51 is able to interact with calmodulin in the ratio of two mols peptide to one mol calmodulin, indicating two separate interaction sites. In fact, one of their fragments interacts with calmodulin in the ratio of two mols peptide to one mol calmodulin. In the case of caldesmon, stoichiometry of interaction with calmodulin is believed to be 1 : 1 (Shirinsky *et al.*, 1988) suggesting that one molecule of caldesmon interacts with two sites on one molecule of calmodulin simultaneously.

The major interaction of calponin by which it is believed to exert an effect is the actin-calponin interaction, which is an area which still requires physical

characterization. It has been shown that calponin can interact with actin, and that by virtue of this ability, calponin is able to inhibit the actin activated Mg^{2+}-ATPase of myosin. Since calcium binding proteins are able to reverse this inhibition, this interaction between calponin and the calcium binding proteins may well interfere with calponin's interaction with actin. Through biophysical studies, the relationship between these three proteins could be investigated in order to more fully understand the system. Experiments such as solid phase interaction studies using ELISA type assays or cosedimentation assays would elucidate competition between these proteins, and offer clues as to how they may interact in a physiological situation. In addition, mutants of calponin could be used with this same methodology to determine the necessary sequences for calponin to bind to actin, for calcium binding proteins to reverse this interaction, and subsequently to delineate the functions of each of the separate calcium binding protein sites if they could be selectively eliminated. In order to provide further information on the sites of interaction of calponin with calcium binding proteins, peptides of the highly exposed regions of calponin which are proposed to be the interaction sites could be synthesized in order to determine the ability of these smaller fragments to interact with calcium binding proteins, and these peptides could be used as tools to investigate calponin's function in biological assays since they may well interfere with the ability of a given calcium binding protein to regulate calponin without preventing calponin's inhibition of ATPase. The chemically produced fragment CP 2-51, along with the mutants provided in the collaborative studies have not been

utilized to their full potential. The effect of these mutants upon ATPase, and the ability of calcium binding proteins to reverse this inhibition will offer insight into the biological function of each of the calcium binding protein interaction sites.

There have been a number of reports that calponin may be localized to the cytoskeleton more than to the contractile domain of smooth muscle cells, and this raises the issue of the role calponin may be playing in the cytoskeleton (North *et al.*, 1994). Certainly if calponin is an actin binding protein of the cytoskeleton, it may inhibit the actin activation of myosin ATPase simply by virtue of its actin binding abilities, without this being its primary role. This was reemphasized when a group proposed that nebulin may be a regulatory protein of skeletal muscle. In this case nebulin, a protein which is believed to contribute to the architecture of skeletal muscle thin filaments by regulating length (Pfuhl *et al.,* 1994), can inhibit the actin activated myosin ATPase, a process which is rendered reversible by calcium calmodulin (Root & Wang, 1994). It is possible that this is a common theme in which actin binding proteins can interfere with other functions of actin, in addition to the one which is their primary purpose.

The discovery that calponin's function can be regulated by caltropin was fortuitous since caltropin was first isolated in this lab. It has been demonstrated in this thesis that calponin can interact with a variety of calcium binding proteins, and an open mind must be kept as to whether caltropin is the true regulator of calponin function *in vivo*. Further to this is the similarity of caltropin to calcyclin, (this lab, unpublished results), a protein believed to be involved in cell cycle

regulation (Calabretta *et al.*, 1986). The ability of calponin to bind so tightly to the S100 family of proteins that play a role outside of thin filament regulation in contractile behavior, i.e. signal transduction, cell cycling, opens many doors to possible roles calponin may be playing in the cell.

In order to determine if we could identify another biological function for calponin in addition to its ability to inhibit the ATPase, we began experiments to find if calponin could exert a direct effect upon actin, *i.e.* affect its ability to polymerize. In order to determine this we have monitored the polymerization of actin by two techniques: fluorescence of actin labelled with acrylodan specifically at cysteine 374; and increase in viscosity as actin polymerizes. It had been reported that calponin was able to affect the fluorescence of pyrenyl labelled actin, returning the fluorescent properties to those characteristic of the globular actin state (Noda *et al.*, 1992). These authors, however, were unable to support a role for calponin in depolymerizing actin with flow birefringence experiments. In our experiments with acrylodan actin, we were unable to detect any effect of calponin upon the rate or extent of polymerization of actin, unlike DNAse, which was able to dramatically interfere with polymerization of acrylodan actin. In addition viscosity experiments were performed, and while S1 was able to increase the viscosity of actin, calponin was again unable to exert an effect. The conclusion from this preliminary study is that calponin does not have a direct effect upon the actin state, however it is able to modulate other actin interactions. In the future the lab will continue experiments to identify other potential roles for calponin by examining its

effect on actin interactions with proteins such as gelsolin, or a cytoskeletal protein such as filamin, and determine if calponin's ability to interact with calcium binding proteins modulates these interactions.

In the study performed to determine if calponin interacted with actin in a similar fashion to other actin binding proteins, it was shown that modification of the actin amino terminus with AEANA did not prevent the ability of calponin to bind to actin, but rather calponin bound to the carboxyl terminus of actin (Miki *et al.*, 1992). Crosslinking studies between actin and calponin localized a crosslinked fragment of calponin to a carboxyl terminal peptide which would support this finding (Mezgueldi *et al.*, 1992). In the crystal structure, however, the amino and carboxyl terminals are very close together (Kabsch *et al.*, 1990), however, and it seems possible that the modifications used may not have been sufficient to rule out the possibility that calponin may bind to the amino terminus in addition to the carboxyl terminus. Further studies are therfore required to identify the regions of actin with which calponin interacts and this will aid in giving insight into why calponin seems to modifiy the effect of other actin binding proteins upon actin, rather than having a direct effect upon actin polymer structure itself. Toward this end, studies have been published in the skeletal muscle system, identifying the amino terminal residues responsible for interaction with TNI through the use of synthetic peptides (Van Eyk *et al.*, 1991) Through the use of amino and carboxyl terminal peptides of actin, it could be established if calponin interacts with one or either terminal end of actin and additionally, analogues of these peptides could

delineate the important residues. This would aid in comparing the behavior of calponin versus other actin binding proteins.

A technique which would lend itself very well to the direction these studies have already taken would be to use tryptophan analogues with altered fluorescent properties that would be included in the protein through molecular biology recombinant techniques, in order to further probe the response of calponin to interaction with a variety of proteins. One type of tryptophan analogue is non fluorescent, while a second type shifts the spectrum of the tryptophan outside the envelope of normal emission [Hogue *et al.* (1992), Bronskill & Wong (1988)]. Since whole calponin does not provide a significant tryptophan response upon interaction, this could be due to the multiple tryptophans within the molecule, and they may be compensating for each other's response. In the case of the mutant CP1-228, it was shown that the tryptophan response was sigmoidal in shape suggesting multiple responses to the stimuli. By replacement of individual tryptophans with synthetic analogues two approaches could be taken. It would allow individual tryptophan responses to be monitored, each tryptophan of which appears to be within a domain of interaction with calcium binding proteins, and the individual responses may provide more information than trying to deconvolute the multiple tryptophan responses. Also, replacement of the tryptophans with analogues of shifted spectral properties would allow the study to focus upon the response of the tryptophan of calponin even in a more complicated system than caltropin-calponin because it would allow the introduction of proteins containing

tryptophan and this would not interfere with monitoring the tryptophan of calponin alone. For instance actin, which also contains tryptophan, could be added in, and the fluoresence of calponin in the presence of actin could be monitored as well as that incurred when calcium binding proteins are added.

Thus, the several proteins that have been isolated from smooth muscle and assigned putative regulatory roles have at least three potential roles in contraction: regulation of actin filament structure as suggested for the function of nebulin in skeletal muscle; formation of the latch state whereby force maintainance in smooth muscle is associated with myosin dephosphorylation and altered crossbridge cycling; or organization of actin and myosin filaments into a coordinated contractile network which may involve a cytoskeletal localization. The challenge for the future is assigning the appropriate roles for each of the components of smooth muscle.

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